Hematopoietic development and immunological tolerance

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Abbreviations

AICD activation induced cell death
ANA anti-nuclear autoantibodies
APC antigen presenting cell
BM bone marrow
cDC conventional DC
CLP common lymphoid progenitor
CMP common myeloid progenitor
cTEC cortical thymic epithelial cells
DC dendritic cell
DLL1 Notch ligand delta-like 1
DLL4 Notch ligand delta-like 4
DN1-4 CD4 CD8 double negative subpopulations 1-4 in the thymus
DP CD4 CD8 double positive subpopulation in the thymus
EKLF erythroid Kruppel-like factor
EPLM early progenitor with lymphoid and myeloid potential
ETP early thymic progenitor
HA hemagglutinin from influenza virus
HCL Hairy cell leukemia
HSC hematopoietic stem cell
ISP immature single positive CD8 subpopulation in the thymus
ITAM immunoreceptor tyrosine-based activation motifs
LSK lineage- Sca1+ c-kit+ hematopoietic precursor
MPP multi-potent progenitor
mTEC medullary thymic epithelial cells
pDC plasmacytoid DC
pTreg in the periphery induced Treg
SCF stem cell factor
SP CD4 or CD8 single positive population in the thymus
Treg regulatory T cell
tTreg thymus-derived Treg
Tx thymectomized

General Summary

The immune system can discriminate between self and non-self, thus assuring clearance of infections by both innate and adaptive effector cells without auto-aggression, thus, establishing tolerance. Projects discussed within this thesis focus on hematopoiesis with special emphasis on T cell development and acqusition of self-tolerance.

The set-up of a protective lymphocyte repertoire commences, in adults, in the bone marrow. A multitude of signals derived from cell-cell interactions and/or cytokine signaling determine the first developmental steps of HSC's differentiating towards the lymphoid lineage. Many cytokines in the BM, e.g. IL-7 allowing proliferation of early B and DC precursor subsets, seem to work in a permissive way. Instructive cytokine signaling that drives cells into differentiation towards the next progenitor stage or a specified cell type by actively switching genes on or off is rarely known. Our data from project I, introduces FLT3L as a cytokine with such an effect. FLT3L was shown to have a direct negative impact on erythropoiesis and, in addition, to drive development of multi-potent progenitors into the myeloid/lymphoid lineages. Therefore, alteration in FLT3L levels affects the balance between erythrocyte, myeloid and lymphoid cell development. Both, myeloid and lymphoid progenitors express the FLT3L receptor (FLT3) and deletion of either the receptor or its ligand leads to defective developmental potential of hematopoietic progenitors. In the present study it was found that, *in vivo* administration of FLT3L promotes expansion of progenitors, which have a combined myeloid and lymphoid potential. Additionally, transgenic mice expressing high levels of human FLT3L were generated to investigate the role of FLT3L overexpression in hematopoietic development. In FLT3L transgenic mice we observed a dramatic expansion of dendritic and myeloid cells, which lead to splenomegaly and blood leukocytosis. Both myeloid and lymphoid progenitors in the bone marrow were significantly increased in numbers with full retention of their normal developmental potential. Furthermore, transgenic mice developed anemia together with a reduction in blood platelet numbers. This finding could be confirmed by FLT3L injection into wild-type mice, which resulted in a rapid

reduction in erythrocyte numbers. All findings together suggest, that FLT3L acts on multi-potent progenitors in an instructive way, inducing their development into myeloid/lymphoid lineages while suppressing their megakaryocyte/erythrocyte potential.

However, other cytokines might become limiting as shown for B cells; here progenitors were reduced in BM likely due to a shortage of IL-7. Instructive cytokines might be useful in therapy to increase transiently a certain cell type for a particular purpose. Thus, for example, FLT3L administration results in an increase of pDCs producing IFN-α, which might be one strategy to combat certain viral infections as well as some malignancies, e.g. HCL. However, increasing IFN-α levels systemically may trigger autoimmune disease, e.g. psoriasis, and therefore therapy approaches are challenging.

Since within this study, we showed a positive effect of FLT3L overexpression on myeloid/lymphoid lineage progenitors, one could assume that thymic T cell development might be directly affected. However, we did not observe alterations in T cell development or an overall increase of the T cell compartment due to transgenic overexpression of FLT3L. It might be that the thymic niche for ETP's is limited in size and therefore, only a specified number of lymphoid progenitors might be able to settle in the thymus. Thus, an increase in lymphoid progenitors in the BM would not affect T cell development due to niche limitations.

The aim of project II was to set up an *in vitro* T cell culture system which is not based on OP9-DL1 or OP9-DL4 stromal cell lines for studying early T cell development prior to T cell selection. Notch-ligand expression by OP9 stromal cells may vary; thus, reproducibility of culture conditions has been proven difficult. To address timing, durability and reversibility of Notch signaling effects on hematopoietic precursors, the "plastic thymus" *in vitro* culture system was developed. To provide precursors with an adequate signal, a DLL4-Fc fusion protein is immobilized at the surface of cell culture flasks. The Fc-part of the fusion molecule is fixed to the surface by means of a monoclonal IgG anti-Fc antibody, thus directing the orientation of DLL4 towards the cells. We have studied the ability of surface-bound DLL4-Fc to induce prolonged and extensive expansion of *ex vivo* isolated $Sca1^+$ $c\text{-}kit^+$ fetal liver

or adult BM haematopoietic progentiors (LSK's). Functionality of *in vitro* generated pro T cells could be demonstrated by transplantation experiments. "Plastic thymus" derived pro T cells could be shown to reconstitute the thymus of irradiated $CD3^{-/-}$, $preTalpha^{-/-}$ or WT mice and generate a functional peripheral T cell compartment of both $CD8^+$ and $CD4^+$ antigen reactive T cells.

The ability to differentiate and culture T cell progenitors starting with $ScaI^+$ $c\text{-}kit^+$ BM cells could become a promising research tool and might even be adapted for clinical applications. Patients suffering from diseases leading to low T cell frequencies, as e.g. HIV infected patients, often suffer from infections, which in people with a functional T cell system usually are easily cleared. However, for final T cell development a functional thymus is needed, thus, this approach applied for the clinic implies that potential patients possess a normal differentiated thymus. Furthermore, the presence of Treg cells is a prerequisite since freshly differentiated T cells otherwise will show uncontrolled proliferation upon activation, which eventually might lead to autoimmunity. Thus, we are currently investigating systems in which mice are coinjected with either sorted $CD25^{high}$ WT Treg's or FoxP3 transduced proT cells to increase Treg levels at early time points.

Autoimmune diseases develop when self-reactive T cells that escaped negative selection initiate a harmful immune response against self-antigens. However, factors, which influence the initiation and progression of an autoimmune response, remain incompletely understood. In order to study the influence of the antigen expression level on central and peripheral tolerance, transgenic mice showing different expression levels of the neo-self antigen hemagglutinin from influenza virus (HA) were generated. The neo-self antigen HA was expressed under control of the CD11c promoter together with a CD4 T cell derived TCR specifically recognizing that HA antigen in the context of the MHC molecule $I\text{-}E^D$. Differences in HA expression levels dramatically affected the set up for T cell tolerance. In both tested double transgenic mice, characterized by either low or high expression level of HA antigen, HA reactive T cells could escape negative selection by expression of a second endogenous TCR α-chain. Thus, T cells expressing two TCR's at the same time could be identified in the periphery of these mice.

In the thymus, these cells could be selected into the T cell repertoire due to an altered affinity to the HA antigen or due to a different specificity. Surprisingly, the resulting phenotype among mice expressing low or high levels of HA antigen differed dramatically. While moderate self-antigen expression levels favored the development of self-antigen-specific regulatory T cells and established a tolerogenic environment, high dose of antigen expression resulted in a very stringent negative selection process and in poor development of self-antigen-specific regulatory T cells. In animals with very low or practically not detectable levels of Treg cells, especially at a very early age, animals suffer from a dramatic disease phenotype characterized by an early onset of anemia and splenomegaly, and the late development of arthritis accompanied by high titers of IgG anti-nuclear autoantibodies. In such a setting, T cells will get strongly activated due to the high antigen expression level in the periphery. A lower antigen expression level could be shown to drive T cell activation and proliferation *in vitro*, however not to the extent observed with a high antigen load. It was shown that a high affinity is needed to fully activate T cells and to drive proliferation. In that respective study affinity but not avidity was taken into account, and it could well be that in case of a given affinity between TCR and antigenic peptide, avidity decides about the actual outcome. The observation that Treg cells recognize the respective (self-) antigen with a high affinity and that the number of antigen specific T cells correlates inversely with the number of antigen specific Treg cells proposes that Treg development depends on strong TCR-mediated signaling. Very likely a strong signal can be mediated in different ways: a high affinity will lead to a long and intense interaction between T cell and APC, however it is conceivable that many short-lived interactions which resemble a high avidity might lead to a similar outcome. Probably it is a combination of both affinity and avidity, which decides about cell fate during T cell development.

Transfer of antigen-specific regulatory T cells into double transgenic newborns expressing the neo-self antigen on a high level ameliorated the early onset signs of disease but could not prevent the development of long-term chronic pathologies. It might well be that injected Treg cells at one point get exhausted and thus, lose their therapeutic effect. Another explanation could be that injected Treg cells might become outnumbered, since the thymus in that system constantly releases autoreactive

T cells into the periphery where they become activated. These findings might point out limitations of Treg based therapy.

The incidence of autoimmune disease in man increases with age. In project IV, the findings of high titers of anti-nuclear autoantibodies (ANA) in more than 80% of 10 months old C57BL/6 (B6) mice, immune complex deposition in glomeruli of kidneys as well as lymphocyte infiltrations in salivary glands, resemble this human phenotype. It is remarkable that the phenotype of these mice was not only determined by activated and infiltrating T cells, but as well by a T cell dependent IgG ANA generation, since MHC class II deficient B6 mice failed to produce IgG ANA. Due to the fact that IgG ANA production in non-aged mice could be induced by adoptive transfer of radio-resistant T cells derived from aged IgG ANA positive mice, it looks like B cell selection processes in the BM are not very stringent. Those B cells, which will generate IgG ANA's at a later stage in life, seem to be present already at young age, however to be activated, autoreactive T cells are needed which only occur in aged individuals.

Thymectomy of 5-week-old B6 mice accelerated the onset of the autoimmune phenotype seen in old B6 mice. Thus, the findings in this study indicate, that disturbed T cell homeostasis may drive the onset of some autoimmune symptoms, like ANA production. Therefore both, a tolerant T cell repertoire as well as T cell homeostasis, seem to be superior prerequisites for the set-up of tolerance. This assumption could be strengthened by the observation that reconstitution of T cell deficient mice with T cell progenitors derived from an *in vitro* culture system (the so-called plastic thymus described in project II) lead to the development of IgG ANA's over time. Thus, one single wave of developing T cells can trigger the onset of that autoimmune feature.

Spontaneous IgG ANA production was not only dependent on age but as well on the genetic background of the mice. Whereas B6 mice were prone to IgG ANA production, mice of the DBA/2 strain were not. However, BDF1 mice, an intercross between the B6 and the DBA/2 strains, showed an intermediate incidence of IgG ANA generation.

In old B6 mice the T cell phenotype was shifted towards a memory phenotype. Increased levels of IFN-γ and IL-2 in aged B6 mice indicated, that T cells are activated, even though old B6 mice show increased Treg levels compared to young counterparts. This gain can very likely be explained by the increased IL-2 availability in such an autoimmunity-prone setting. However, no obvious therapeutic effect could be observed.

To summarize, the set-up of T cell tolerance is important to circumvent immediate T cell derived autoimmune features. Additionally, there is evidence that a tightly controlled homeostatic T cell repertoire is needed to ensure B cell tolerance.

Introduction

1. Lymphocyte development

Models of hematopoiesis

In adults, hematopoetic stem cells (HSC) home to the stem cell niches in the bone marrow where they remain life-long in an undifferentiated stage until a differentiation signal allows HSCs to leave their niche and to differentiate. All hematopoietic lineages develop via intermediate multi-potent progenitor cells. The classical model for hematopoiesis is the Weissman model [1, 2], which proposes a hierarchical development of hematopoietic lineages. According to this model developing cells gradually loose their multi-potency. At the branching point for the postulated common myeloid (CMP) or common lymphoid progenitors (CLP), the multi-potent progenitors (MPPs) eventually have to commit themselves irreversibly to the lymphoid or the myeloid lineage. However, this model was refined when the high plasticity of Pax5-deficient pro B cell clones could be shown by reconstitution of both myeloid and lymphoid lineages *in vitro* and *in vivo* respectively [3-5]. In 2005, the *in vivo* equivalent of the Pax5-deficient pro-B cell was discovered – an early progenitor with lymphoid and myeloid potential (EPLM) that equals the pre-pro B cell in mouse BM [6, 7]. EPLM (as *Pax5*$^{-/-}$ pro B cells) express B220 and c-kit and are negative for CD19 and NK1.1. Furthermore, EPLM express IL-7Rα (CD127), Flt3 (CD135) and CD93. The plasticity of EPLMs led to the proposal of the pairwise relationship model of hematopoiesis. This model describes hematopoiesis as "a series of invariant pairwise developmental relationships between the various hematopoietic lineages" [8]. Thus, there are several different intermediate progenitors that may differentiate towards one final cell fate. While progressing in development, these progenitors seem to keep a certain degree of their plasticity, that allows reversing cell fate choices at different developmental levels. In 2005, Rolink and colleagues described CD117^{+} DN1 cells, the earliest subpopulation of thymic precursors, giving rise also to myeloid cell lineage development and thus, confirmed the pairwise relationship model of hematopoiesis [6]. This finding could be assured in 2008 by Bell and Bhandoola [9].

FLT3L in hematopoiesis

Hematopoieses is a highly complex and tightly regulated process. Several transcription factors (e.g. Pax5, EKLF, GATA-1, AP-1), signaling proteins, adhesion molecules and cytokine receptors are known factors, that are involved in regulating hematopoiesis. Several cytokines, e.g. IL-7, were identified as crucial factors for generation of different hematopoietic lineages due to their ability to impart environmental signals into hematopoietic development. Recently, in our laboratory, the positive role of Flt3L in dendritic cell (DC) development and its importance for survival and expansion of lymphoid and myeloid progenitors could be shown (manuscript in preparation). When HSC's are provided with sufficient FLT3L signal, they will predominantly give rise to lympho-myeloid progenitors. In contrast, HSC's that only receive a weak FLT3L signal due to either low FLT3 expression or low FLT3L levels in the microenvironment will develop into megakaryocyte-erythrocyte progenitors (manuscript in preparation). Myeloid, erythroid and B cell lineages differentiate within the BM as one of the two primary lymphoid organs. Early thymic progenitors (ETPs) leave the BM at an early progenitor stage and enter via blood vessels [10] the thymus – which is the second primary lymphoid organ - for differentiation into mature T cells.

2. T cell development

Early T cell development

The earliest T cell precursor found in the thymus is the ETP. ETPs are defined as Lin^- c-Kit^+ $Flt3^+$ $CD24^{-/lo}$ $CD44^+$ $CD25^-$. Their frequency is about 0.01% of all thymocytes. To keep a functional T cell repertoire, the thymus has to be colonized constantly with BM derived ETP's [10]. Ceredig *et al* proposed that about five bone marrow-derived precursors per day colonize the thymus and hence, sustain T cell development [11]. Therefore, the thymus needs to provide, for instance, chemokines to attract progenitor cells, survival factors and specification signals to induce and maintain differentiation towards distinct cell fates. Recently, it was postulated that there are four main factors necessary for the set up of an environment in which T cell development can take place efficiently. The factors are the chemokines CCL25 and CXCL12, the cytokine stem cell factor (SCF) and the Notch ligand Delta-like 4 (DLL4) which all are all absent in

the primitive thymus anlage in fetal mice deficient of FoxN1 transcription factor [12]. CCL25 and CXCL12 are important for the regulation of homing of hematopoietic precursors to the fetal thymus [12, 13]. In addition, CXCL12 was shown to increase efficiency of the transition from DN3 to DP in adult mice (DN3 - subset of CD4 and CD8 double negative population in the thymus; DP - subset of CD4 and CD8 double positive population in the thymus; detailed description will follow) [14]. Notch signaling via Notch 1 and one of its ligands, DLL4, is crucial for the induction of T cell development. Conditional inactivation of the receptor [15] or DL4 [16, 17] results in complete abrogation of T cell development. Furthermore, it could be shown that in the absence of DLL4 - dependent on the presence of SCF and/or Cxcl12 – only myeloid and/or B cells develop in the thymus [12]. Thus, DLL4 signaling is key for T cell development and has highest priority within the four listed signaling pathways [12]. Another important factor for T cell development in adult mice is the cytokine IL-7. The contribution of IL-7 was not addressed in the study mentioned above, since IL-7 was still found to be present in the primitive thymus anlage in fetal mice deficient of FoxN1 transcription factor. Additionally, it was shown that fetal T cell development is IL-7 independent [18]. However, for T and B cell development in adult mice, IL-7 is a critical cytokine since IL-7 deficient mice exhibit severe lymphoid abnormalities [19]. Analysis of CD127 expression, the α-chain of the IL-7R, revealed only a very weak signal on thymocytes up to the DN2 stage (subset of CD4 and CD8 double negative population in the thymus) using the monoclonal antibody (mAb) A7R34. However, it could be shown that *in vitro*, ETP and DN1 (subset of CD4 and CD8 double negative population) survival and growth is strictly dependent on IL-7 [20]. Furthermore, IL-7R signaling was shown to be critical, not only in early thymocyte expansion prior to T cell receptor gene rearrangement, but as well for proliferation of early B cell progenitors undergoing immunoglobulin heavy chain gene rearrangements [21]. Besides these findings, a blocking activity of IL-7 during the transition of DN3 (subset of CD4 and CD8 double negative population) to the DP (CD4 and CD8 double positive population in the thymus) stage could be shown using *in vitro* assays. Only upon withdrawal of IL-7 cells differentiate further into DPs [14, 20]. However, it is worth mentioning, that progenitors isolated from adult mice were more susceptible to inhibition of differentiation than fetal liver derived progenitors [20] suggesting a difference in lymphoid development comparing

fetal and adult progenitors. Furthermore, IL-7 was reported to play a role in the development of the CD8 T cell lineage [22, 23]. Summing up, the role of IL-7 in T cell development is not quite understood and recent *in vitro* findings seem contradicting concerning a positive or negative impact of IL-7 on T cell development. Nevertheless IL-7 seems to play a crucial role in thymic developmental processes.

Developmental stages in T cell development

Based on the surface expression of the TCR co-receptors CD4 and CD8, developing thymocytes can be subdivided into four major populations. In the developmentally earliest stage, thymocytes are $CD4^-$ and $CD8^-$ and therefore called double negative (DN). The maturity of DNs can be examined by analysis of CD25, CD44 and CD117 expression. According to their differential expression levels one can distinguish four different populations; DN1 to DN4. DN1 cells are characterized by high surface expression levels of CD44 and CD117, while they are negative for CD25. This stage is followed by the DN2 stage, where cells express all three surface markers. High levels of CD25 and low levels of both CD44 and CD117 are characteristic for the DN3 population [24-26]. At this stage of development, cells complete the rearrangement of their β-chain locus. Once, T cell progenitors express a successfully rearranged β-chain, there is a negative feedback-loop to stop further rearrangement events of this locus and thus, only one locus is rearranged and expressed as protein. This procedure is known as allelic exclusion at the TCR β-locus [27, 28]. Developing T cells are selected in case of a productive rearrangement in a process called β-selection. For being selected DN3 cells with a functional β-chain rearrangement express the β-chain together with the pre Tα-chain on the surface [29, 30]. Due to preTCR-mediated proliferation, DN3 cells expand and eventually differentiate into DN4 cells. DN4 cells loose expression of CD25, CD44 and CD117 and directly differentiate further into so-called immature single positive (ISP) CD8 cells [24-26] in mouse. (In humans there is a similar immature single positive stage, however instead of CD8, CD4 is expressed.) ISPs differentiate further into CD4 and CD8 expressing cells that are called double positive thymocytes (DP). At the double positive stage, the TCR α-locus will rearrange. For the α-locus, there is no allelic exclusion observed. Thus, α-chain rearrangement only will terminate as soon as an α-chain is produced that can pair with the expressed β-chain and provide the cell with a functional signal. This signal eventually composes the end point of all rearrangement events at the αβ-

TCR locus. As soon as DPs express a functional αβ-TCR on their surface, cells undergo positive and negative selection. Thus, only DPs that receive a survival signal due to a functional TCR and DPs that are not deleted due to a too strong signal caused by high affinity for self antigens, will be able to differentiate further [31, 32]. In this stage, most thymocytes die by neglect due to non-functional rearrangements. Depending on the class of MHC molecule, which can be recognized by the TCR, thymocytes become committed either to the CD4 (TCR-MHC II interaction) single positive (SP) or to CD8 (TCR-MHC I interaction) SP T cell lineage. Selected and hence self-tolerant CD4 and CD8 SPs are able to leave the thymus through the efferent blood vessels and scan for presented antigens in secondary lymphoid organs.

T cell selection

During T cell development T cells get selected in order to provide the host with a powerful T cell repertoire to recognize foreign antigens but which is at the same time tolerant to self. The selection procedure is known as positive and negative selection. For being positively selected T cells need to be able to interact with self-MHC to receive a survival signal [33]. Without this signal T cells die by neglect. Negative selection is based on the recognition of self-peptides presented by MHC molecules in the thymus; i.e. T cells that receive a strong signal in the thymus will undergo apoptosis [34]. Even though the outcome of these selection processes seems to be clear, the actual signaling is not well understood. The affinity with which the TCR recognizes the peptide presented by an APC on the MHC molecule was shown to play a major role during selection of CD8 positive cells. The finding that there seems to be a fixed affinity threshold for negative selection suggests that negative selection – at least for CD8 T cells - depends on differences in affinity [35]. High affinity interactions will lead to a strong signal due to a prolonged interaction of TCR with the respective peptide presenting MHC molecule. This prolonged interaction might result in a level of phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3 complex that is necessary to initiate negative selection [36]. However, there is evidence, that affinity is not the only important factor. Avidity, which means the sum of all TCR-APC interactions, can modulate selection procedures. Our findings derived from double transgenic mice expressing the HA antigen under the CD11c promoter and in addition a transgenic HA-reactive TCR on CD4 positive T cells, support this reasoning (manuscript in preparation). Using the

HA double transgenic mouse model, we could show that a low expression level of peptide on the surface of APCs leads to a completely different outcome compared to a high expression level. Negative selection of CD4 positive HA-reactive T cells itself, seems not to be altered severely by differential expression levels of the neo-self-antigen. However, regulatory T cell levels decreased dramatically in case of high antigen expression in the thymus. Thus, avidity, which is studied here in context of CD4 T cells, appears to have a major influence on the set-up of tolerance. Aschenbrenner *et al* (2010) had similar findings. In their study, due to a decrease of MHCII-dependent antigen presentation on TECs, they could observe percentagewise an increase in the respective antigen-specific Treg population [37, 38]. Up to date, it is discussed whether different APC subsets have specialized function, or whether it is the signal strength on its own that decides the outcome of the selection process.

In vitro T cell development

The three dimensional network of thymic stromal cells has a major implication on T cell development. It provides developing thymocytes with a multitude of exogenous, indispensible signals and a customized microenvironment. Even though, *in vitro* and *in vivo* approaches are described to study detailed processes of T cell development, the initiation of this process and the time point upon which T cells are absolutely committed to the T cell lineage, as well as the signals for differentiating further towards the next thymic developmental stage, are not clarified yet. In 2002, Schmitt and Zuniga-Pflucker [39] published the first two dimensional *in vitro* culturing system for developing T cells based on Delta-like 1 (DLL1) expressing OP-9 stroma cell line. The possibility to differentiate T cells *in vitro* opened up new opportunities to study T cell development in more detail. However, there are several disadvantages using this stroma cell based system, e.g. variation in stromal cell Notch ligand surface expression due to several cell passages or varying culturing conditions and unknown signaling components provided by stroma cells. Ohishi *et al* [40] described a feeder-free system: DLL1 was expressed with a human Fc-tag as a fusion protein and coupled to plastic surfaces. Using this system, T cell development could be induced in a DLL1 concentration dependent manner [41]. Later on, it was shown that the physiologic thymic Notch ligand responsible for T-cell development is DLL4 instead of DLL1 [16, 17]. To overcome previous *in vitro* culture problems, a T cell culture system was established in our laboratory – the so-called “plastic thymus” (manuscript

in preparation)[14]. In this stromal cell-free system an approach similar to Ohishi is used, i.e. the fusion protein DLL4-human IgG_1-FC is coupled to a tissue culture plate pre-coated with monoclonal anti-human IgG_1-Fc antibody (Huf 5.4). With this culture system, a powerful tool was generated to study minimal requirements for T cell development where conditions can be manipulated easily and in a well controlled manner.

Antigen presenting cells in the thymus

T cell development in the "plastic thymus" is terminated at the DP stage. For T cell development beyond this stage antigen presenting cells (APCs) are needed for T cell selection processes. The urge for thymic T cell selection is obvious, auto-reactive T cells are deleted and T cells that are not capable to interact with self-MHC molecules die by neglect. Antigen presenting cells in the thymus include cortical (cTEC) and medullary thymic epithelial cells (mTEC) as well as dendritic cells (DC) and B cells, however B cells are only present as a tiny population (about 1% of the starting thymus suspension) in the thymus compared to the other APC lineages [42, 43]. cTECs are localized in the outer cortical regions of the thymus. cTECs were shown to be able to positively select T cells [44, 45]. Besides that, there is some evidence that cTECs also might be able to contribute to negative selection [46, 47] as well as to Treg induction [48]. The mechanism how cTECs contribute to T cell selection is unknown. One of the first models discussed in the field is the "altered peptide model". cTECs were suggested to present "specially tailored" peptides for positive selection . Recently, there is evidence that the peptide machinery in cTECs, indeed, differs from proteolytic pathways in other cell types, e.g. cTECs preferentially express cathepsin L instead of cathepsin S for the degradation of the invariant chain in the MHCII loading process [49]. Furthermore, macroautophagy seems to play an important role for the cTEC function [50]. cTECs express high levels of surface MHCII, but are particularly inefficient to present exogenous proteins using the classical endocytic pathway [51, 52]. It could be demonstrated, that via macroautophagy, intracellular antigens can be delivered to the MHC II pathway and, thus, shape the $CD4^+$ T cell compartment [53]. Last but not least, cTECs were shown to express a different subunit in the proteasome - β5t instead of β5i – and therefore, protein processing is different compared to mTECs and DCs [54]. This finding supports the idea, that the peptide repertoire presented by cTECs differs from the one presented by mTECs and DCs. Other models

claim, that selection is based on affinity or avidity, hence taking both into account, the quality and the quantity during the interaction of MHC presented antigen and TCR (refer as well to above). It is very likely, that neither model is correct, and that the truth lies in a combination of the two. Subsequent to CD4 or CD8 lineage commitment, thymocytes translocate to the medulla where they reside up to 5 days prior to exiting the thymus [55]. In the medulla developing T cells undergo a quality check. By interacting with mTECs and DCs, self-tolerance of T cells is assured. In case of a premature egress of thymocytes or disorganization of the medullary architecture, systemic autoimmunity is observed [56, 57]. This shows, that T cell deletion during thymic negative selection is a crucial requirement for the set-up of central tolerance. mTECs, together with cTECs, are the only non-hematopoietic cells with a constitutively high MHC II expression [43]. mTECs were shown to present tissue-restricted antigens in the thymus, in part under the control of the autoimmune regulator transcription factor AIRE [58]. Their contribution in deletion of auto-reactive T cells is without controversy [52]. Additionally, mTECs were shown to be involved in thymic Treg generation [37].

The dendritic cell (DC) is another important antigen presenting cell type in the thymic medulla. Thymic DCs can be subdivided into three major subsets: conventional $CD11c^{high}$ DCs (cDCs) that are $CD11b^{-}CD8\alpha^{+}CD172a^{-}$ or $CD11b^{+}CD8\alpha^{-/low}CD172a^{+}$ and $CD11c^{mid}CD45RA^{+}$ plasmacytoid DCs (pDCs) (reviewed in [59]). Only the $CD11b^{-}CD8\alpha^{+}CD172a^{-}$ cDCs, that represent approximately two thirds of the cDC compartment, arise in the thymus (autochthonous). $CD11b^{+}CD8\alpha^{-/low}CD172a^{+}$ cDCs, as well as pDCs, immigrate from periphery into the thymus and therefore are called migratory DCs [60-62]. According to a study where several steady-state splenic DC subsets were injected intravenously and their homing to the thymus was monitored, all three major DC subsets of peripheral cDCs ($CD11b^{-}CD8\alpha^{+}$ 'lymphoid' DCs, $CD11b^{+}CD8\alpha^{-}$ 'myeloid' DCs and $CD11b^{-}CD8\alpha^{-}$ DCs) showed a similar ability of homing to the thymus and additionally, their contribution to negative selection could be revealed [63]. However, other experimental approaches could show, that under physiological conditions almost all migratory thymic cDCs have myeloid features and most circulating cDCs in the blood express CD172a [62], which is expressed by peripheral myeloid cDCs [64]. Thus, it is very likely that under physiological conditions almost all peripherally derived migratory cDCs in the thymus have a

'myeloid' phenotype – most likely due to their relative abundance in the circulation. After entering the thymus, migratory CD172a$^+$ DCs preferentially localize to the medulla and intermingle with autochthonous DCs. They upregulate MHC II and co-stimulatory molecules, i.e. they undergo further maturation [62]. Interestingly, LPS pretreated and adoptively transferred DCs could not home to the thymus anymore, suggesting that there is a protection mechanism to prevent central tolerance induction towards pathogen-derived antigens taken up in the periphery [63]. In several studies, the contribution of migratory DCs to negative selection could be shown [63, 65]. In the second study, not only partial deletion of the respective antigen-specific T cells could be shown, but, as well, a slight increase in Tregs displaying the very same specificity [65]. However, it is under debate, whether a specialized type of APC is needed for Treg induction or if Treg development is under T cell-intrinsic developmental control. *In vitro* assays showed, that all thymic APC subsets efficiently could promote Treg conversion using immature thymocytes, but not peripheral naïve CD4$^+$ T cells [66]. Based on this, different anatomical DC origins could account for a 'complete' pool of peptides presented in the thymus. Migratory DCs were suggested to sample serum antigens and well-accessible antigens, whereas autochthonous DCs might rather concentrate on antigens that are difficult to be reached by migratory DCs. Furthermore autochthonous DCs were shown to be much more efficient, compared to migratory DCs, in cross-presenting antigens derived from apoptotic cells to CD8$^+$ T cells [61]. In this context, it should be mentioned, that up to date, there is only evidence for a uni-directional antigen transfer from mTECs to DCs [67] but not from DCs to TECs. Thus, tissue restricted antigens promiscuously expressed by mTECs can be presented by DC subsets in the thymus. To sum up, the heterogeneous APC subsets in the thymus derived from different anatomical origins, very likely altogether, account for the presentation of a broad range of self-antigens and hence, they assure the set up of central tolerance.

3. Autoimmunity

Central tolerance and autoimmunity

Development of autoimmune disease can be caused by a huge variety of sources. Failure in the set-up of central tolerance can be one reason that most likely will lead

to autoimmune disease development. These failures can occur at a very early developmental stage. Mutations in the *FoxP3* gene locus is an example for such a scenario. The autoimmune disease phenotype is known as the IPEX syndrome in men (typically caused by missense mutations) or the scurfy phenotype (spontaneous frameshift mutation or complete knockout of the *FoxP3* gene) in mice [68-72]. The transcription factor Foxp3 is important for the generation and function of Tregs. A defect in FoxP3 will hinder the development and the functionality of Treg cells and thus, will lead to autoimmunity [73]. Mutations in the *AIRE* gene locus can also lead to autoimmunity. AIRE is expressed by mTECs in the thymic medulla and is responsible for ubiquitous gene expression, including tissue-specific self-antigens, in the thymus. Due to non-functional AIRE expression, negative selection for T cells is not complete and self-reactive T cells can be released into the periphery causing severe autoimmune diseases (reviewed in [74]). However, failures in the set-up of central tolerance, even though the auto-antigen is expressed in the thymus can occur. In both autoimmune diseases, Myastenia gravis and Diabetes type I, the respective auto-antigen is reported to be expressed in the thymus, however on a different level, compared to healthy controls [75]. Thus, tolerance can be broken not only due to non-expression of the auto-antigen but as well due to deregulated expression levels of the respective self-antigen in the thymus. In contrary, other autoimmune diseases are very likely to develop independently of the thymic selection processes; e.g. rheumatoid arthritis.

Age and autoimmunity

It is a well-accepted observation that several autoimmune diseases occur in the second half of life or peak in the elderly [76, 77]. Rheumatoid as well as giant cell arthritis and Sjögren's syndrome are only few examples [78-80]. Moreover, anti-nuclear autoantibodies (ANA) are frequently found in aged individuals [81, 82]. This phenomenon can be seen as well in aged mice depending on their genetic background (manuscript in preparation). However, up to date, the cellular mechanisms that cause the onset of the development of these autoimmune features are only poorly understood. The competence of the immune system declines with age. Findings like a higher mortality and morbidity upon annual influenza epidemics [83], as well as only limited protection upon influenza vaccinations in the elderly population support this conclusion [84, 85]. With age the production of T and B cells decreases, thus this

decrease is thought to lead to the decline of the immune system's competence. It could be shown that B cell production is reduced with age in mice. Whereas 1 out of 50 pro B cells isolated from 2 week-old mice can be grown on stromal cells in the presence of IL-7 *in vitro*, in aged mice this frequency is dramatically decreased. In aged mice, only 1 out of 500-1000 pro B cells can be cultured and therefore would be able to differentiate into terminally differentiated and functional B cells *in vivo* [86]. In man, the drop in B cell generation with age is even more striking as pro- and pre-B cells are practically undetectable in the bone marrow of individuals that are older than 85 years [87, 88]. There is not only a difference in the early B cell populations comparing young and adult individuals, but, as well, a difference in their phenotype. In adult humans, the peripheral B cell compartment consists to a large extent of memory B cells marked by expression of CD27 (reviewed in [89]). So far, in mice, memory B cells were not reported. In humans, this finding might be due to the fact that the turnover of mature B cells, including memory B cells, is slow or even absent due to the lack of competition for space with newly formed B cells from the BM. The drop in developmental capability is not only restricted to B cells but also includes T cells. T cell generation decreases dramatically with age in mice as well as in humans. Thus, the thymus involutes with time [90]. The mechanism for this involution is not fully understood. The subsequent decrease of newly generated naïve T cells leads to a very low exchange rate of mature peripheral T cells with newly generated ones. Not only the peripheral B cells in adult human beings, but as well peripheral T cells predominantly have a memory phenotype in mouse and man (reviewed in [91]). This might mirror the frequent encounters of the T cell repertoire with foreign antigens in adult individuals. Another explanation might very well be, that the homeostatic proliferation of T cells in adults is increased due to the reduced thymic output. Thus, B and T cell generation are affected by aging.

The finding of a reduced B and T cell development with age seems contradictory to the observation of a higher incidence of autoimmune disease developing in adults. This paradox might lead to the conclusion that both B and T cell selection is impaired in aged individuals, and hence maybe less strict, however there is no real evidence for that reasoning up to date. Another explanation could be that the disturbed turnover, and thus an impaired homeostasis, causes autoimmunity. The findings in our study, referring to T cells, strongly support the second rationale.

The role of genetics in autoimmunity

Besides age and non-functional central tolerance, genetics also play a major role in the development of autoimmune disease. Morbus Bechterew, a disease syndrome also known as ankylosing spondylitis, is strongly associated with the expression of HLA-B27 [92]. The pathogenesis of spondylitis is believed to depend (i) on the HLA-B27 peptide-presenting specificity (absolute restriction for peptide ligands with arginine at position 2 [93]), (ii) on the slow-folding and the high probability for misfolding of the HLA-B27 molecule and/or (iii) on the capacity of HLA-B27 to form covalently linked heavy chain homodimers that can be recognized by leukocyte receptors. Thus, referring to spondylitis, autoimmune disease is believed to be triggered by (i) self-reactive T cells against a self-ligand of HLA-B27 elicited by a cross-reactive foreign antigen, (ii) independently from antigen presentation, as a result of endoplasmatic reticulum stress, and/or (iii) immunomodulation of both the innate and adaptive immune responses to arthritogenic pathogens due to recognition of heavy chain homodimers by leukocyte receptors. Type 1A diabetes is another example of an autoimmune disease for which the genetic background is decisive. HLA alleles DQ and DR were identified as important determinants of disease and even allow to identify at birth individuals at high risk [94].

4. Peripheral tolerance

Anergy

Central tolerance is completed by mechanisms that assure tolerance in the periphery. Although central tolerance is efficient, not all self-antigens are expressed in the thymus. Thus, some lymphocytes only encounter their cognate self-antigen outside of the thymus in the periphery. Food antigens, developmental antigens and antigens displayed during chronic infections are only a few examples of such antigens. Without peripheral tolerance mechanisms, recognition of those antigens by T cells can lead to the onset of autoimmunity. Induction of anergy in the periphery is one important mechanism to keep tolerance. For complete T cell activation, T cells need two signals. First, T cells need to receive a positive signal via their TCR upon recognition of the respective antigen presented by MHC. In addition, T cells depend on a second costimulatory signal derived from the APC, which is mediated, e.g., via

CD28 ligation. Successful antigen recognition is followed by cytokine secretion, such as IL-2. The PI3K/AKT-mTOR pathway is fully activated by subsequent signaling through the IL-2R complex. In case of a missing second signal, T cells remain in a long-term hyporesponsive state, which is termed anergy. Anergic T cells are characterized by active repression of both, TCR signaling and IL-2 expression [95]. mTOR was reported as a sensor for energy and nutrients and it was shown that by blocking mTOR activation, anergy in T cells could be induced. Powell and Degoffe suggested that several energy and nutrient sensing pathways, by measuring nutrient deprivation, ATP deprivation and hypoxa, might induce anergy through mTOR inhibition [96]. Tregs have been proposed to foster a hypoxic environment due to expression of 5'-ectonucleotidase CD73 and ATPase/ADPase CD39 [97, 98]. Both enzymes together convert ATP into adenosine and thus, Tregs may regulate T cell activation by promoting anergy. T cell activation depends on costimulatory signals; however costimulatory pathways can as well provide negative signals that can inhibit T cell responses and mediate tolerance. The programmed death 1 (PD-1) receptor and its ligands PD-L1 and PD-L2 is one example of such a negatively regulating costimulator molecule [99]. Ligation of TCR and PD-1 attenuates the activation of PI3K and Akt pathways through dephosphorylation of the proximal signaling molecules by SHP-1 and SHP-2. CTLA-4 is another important costimulatory molecule in anergy induction. CTLA-4 is induced at a late stage in T cell activation and binds to the B7 proteins CD80 and CD86 with high avidity competing with CD28. However, unlike CD28, CTLA-4 transduces a negative signal preventing cell cycle progression, and thus limits T cell responses. APCs not only induce immune responses but also can induce and maintain tolerance. Tolerogenic DCs present antigen but cannot deliver adequate costimulatory signals to antigen-specific T cells, thus T cell activation and proliferation is hindered [100]. Since the capability to promote tolerance is not restricted to a specific DC subset it is rather believed that tolerogenic DCs are DCs with incomplete maturation. It was shown that apoptotic cells are not able to trigger full DC maturation whereas necrotic cells can do so [101]. Additionally, an immunosuppressive environment (IL-10, TGF-β) supports the generation of tolerogenic DCs [102].

Peripheral deletion

Peripheral deletion of self-reactive lymphocytes is another important mechanism to achieve tolerance. Fas (CD95) is expressed on T cells; upon repeated stimulation by their cognate or foreign antigen and IL-2, FasL (CD178) expression is induced on T cells [103, 104]. The so-called "death receptor" signaling leads to activation induced cell death (AICD) by activation of Caspase-8 and effector caspases which consequently promote apoptosis. Interestingly, the killing of activated T cells during the shutdown of an immune response is not dependent on Fas but on Bim [105, 106]. Bim activates Bax/Bak and thus, leads to the permeabilization of the outer mitochondrial membrane and eventually to apoptosis. It could be shown that Bim-deficient mice spontaneously develop immune complex-glomerulonephritis at old age and that Bim actively is involved in maintenance of hematopoietic homeostasis [107].

Regulatory T cells

The heterogenous population of regulatory T cells is indispensable for providing dominant tolerance. Recently, a new nomenclature was suggested for the different subsets of regulatory T cells by well-known representatives of 'regulatory T cell' research [108]. Although, up-to-date it is impossible to distinguish different regulatory T cell subsets using surface markers, it could be shown that regulatory T cells can be of two different origins. They can be thymus-derived (tTreg), which previously were also called 'natural Treg'; or they can develop in the periphery (pTreg) and so far often were described as 'induced Treg'. These Treg populations found *in vivo*, should be clearly distinguished from *in vitro* induced Treg populations (iTreg) since the significance of *in vitro* findings are under debate. Furthermore, the term 'regulatory T cells' should only be used for defined $FoxP3^+$ $CD4^+$ T cell populations with a substantial suppressive ability.

The first observations that eventually lead to the discovery of Treg cells were done by Nishizuka & Sakakura in 1969 [109]. Thymectomy (Tx) of female mice early in life resulted in infertility due to autoimmune oophoritis, however only in case when the thymus was removed from neonatal animals at day 3 after birth (d3Tx). Several thymus or lymphocyte grafting experiments followed. These experiments finally lead to the conclusion, that disease suppression was achieved by thymus-derived lymphocytes. Furthermore, these experiments provided evidence that these cells

developed in the thymus of the neonate, but were not released into periphery during the first 3 days of life [110].

Later it could be shown that autoimmune oophoritis or gastritis could be transferred into newborns or adult *nu/nu* mice by injection of T cells derived from spleen of d3Tx mice with disease [111, 112]. Further studies verified that the effector and the suppressor T cell populations were $CD4^+$ $CD8^-$ [113, 114]. The finding that removal of suppressor T cells from an otherwise healthy animal initiates autoimmune disease and that reconstitution of the recipient with suppressor T cells could reestablish self-tolerance was a major advance in the field [115]. Until then, suppressor T cells were only known to be a minor population of the $Thy1^+$ $CD5^{high}$ thymocytes. The next major progress in the field was the discovery by Sakaguchi and associates that suppressor T cells express high levels of CD25, the IL-2R α-chain [116, 117]. With this finding suppressor T cells could be further described as a subpopulation of about 10% of the $CD4^+$ thymocytes. In 2003, the transcription factor FoxP3 was demonstrated to have key regulatory function in regulatory T cell development and function in thymus as well as in periphery [72, 73, 118].

The detailed mechanism for tTreg development is still unknown. However, there is evidence that T cells in the thymus enter the Treg lineage upon self-antigen recognition, e.g. there are studies observing an enrichment of $FoxP3^+$ T cells in polyclonal thymocytes bearing superantigen-reactive TCRs [119, 120]. The current consensus concerning thymic T cell selection processes suggest, that a TCR – peptide presenting MHC interaction of intermediate strength results in positive selection of T cells whereas very strong signals would lead to negative selection. However, it is under debate what kind of signal determines signal strength. According to the affinity model, the affinity of a single TCR – peptide loaded MHC interaction, i.e. the quality of this interaction, determines the outcome of selection [34, 36]. However, several studies indicate that avidity might play a major role in thymic selection. Avidity takes all single TCR interactions into account and therefore quantity has higher priority then high quality signals derived from single interactions [121, 122]. There is evidence from *in vitro* [123] and *in vivo* [124] studies, that the number of developing Treg cells correlate inversely with the amount of antigen presented in the thymus. In contrast, increasing the antigen dose results in a higher incidence of concomitant negative selection [125]. Klein and associates very elegantly produced evidence for

the important role of avidity in Treg development. In AIRE-HA x TCR-HA double transgenic mice, the HA antigen is specifically expressed and presented by mTEC. This experimental set-up results in the deletion of about two thirds of HA-specific thymocytes, whereas a substantial fraction of the remaining cells differentiates into the Treg lineage [37]. In this system, MHC II was knocked-down through an RNAi approach. The decrease in HA presentation was shown to lead to a lower incidence of negative selection, but to an increase in Treg differentiation [38]. Altogether, these studies indicate that intermediate avidities favor Treg differentiation over negative selection [126]. Furthermore, developing tTreg seem to be more resistant to apoptosis, since CD27–CD70 co-stimulation in the thymus was shown to rescue those cells from negative selection and thereby promotes tTreg generation [127].

pTreg are naïve $CD4^+$ T cells that in the periphery get committed to the Treg lineage. It is very likely, that converted Treg cells recognize a different set of antigens that cannot be displayed in the thymus, e.g. environmental or food antigens. Several studies support the hypothesis of distinct TCR specifities of pTreg [128, 129]. As for tTreg generation in the thymus, high affinity recognition of the respective antigen seems to be important for pTreg induction. It could be observed that transgenic T cells stimulated with cognate rare high-affinity antigen promoted more efficient FoxP3 induction compared to low affinity stimulation [130]. Furthermore, there is evidence from several *in vitro* studies that conversion can take place when T cells are stimulated suboptimal, i.e. increased CTLA-4 but decreased CD28 signaling, in the presence of TGF-β [131-133]. Suboptimal T cell stimulation goes in parallel with a noninflammatory environment. In such an environment, chronic systemic administration of low-dose foreign antigen, which equals the occurrence of e.g. environmental antigens, was shown to induce antigen-specific pTreg in both TCR transgenic and polyclonal T cell populations [134-137].

Although, Treg cells can be of different developmental origin, the main purpose of all Treg cells most likely is the same – the suppression of immune response to auto-antigens and immune regulation. The understanding of the mechanisms of Treg suppression is only limited. However, the high expression level of CD25, the α-subunit of the high affinity IL-2 receptor, suggests that Treg cells might deprive effector T cells of IL-2. Lack of IL-2 will inhibit effector T cell proliferation and thus, will hinder an immune response [138]. Several other mechanisms mediating T cell

tolerance are proposed. CTLA-4 mediated suppression [139, 140], induction of IL-10 and TGF-β production by DCs [141] or expression of immunosuppressive cytokines by Treg (e.g. IL-10, IL-35, TGF-β; reviewed in [142, 143]) are only few of the suggested mechanisms reviewed by Rudensky and colleagues [144].

Thesis projects

Project I ***In vivo*** **evidence for an instructive role of fms-like tyrosine kinase-3 in hematopoietic development**

Co-author

Project II **Establishment of a stromal cell free culture system that allows the long-term propagation and proliferation of pro T cells, which can be used for the *in vivo* reconstitution of the T cell compartments**

First-co-author

Project III **The amount of self-antigen determines the effector function of murine T cells escaping negative selection**

First co-author

Project IV **The development of autoimmune features in aging mice is closely associated with alterations of the peripheral CD4 T cell compartment**

First author

Results

I

In vivo evidence for an instructive role of fms-like tyrosine kinase-3 in hematopoietic development

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Running title: "Instructive role of FLT3-ligand in hematopoiesis"

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Authorship and disclosures

P.T., R.C. and A.R. designed the overall research, analyzed the data and wrote the manuscript; P.T. performed cell culture experiments and FACS analysis; L.K.S. generated the transgenic mice and performed initial FACS analysis; A.N. and G.C. performed FACS analysis; N.N. and M.K. performed the *in vivo* FLT3L and IL-7 treatment; H.R. performed histology; A.R. performed cell sorting and the *in vivo* transplantations.

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Abstract

Cytokines are essential regulators of hematopoiesis, acting in an instructive or permissive way. Fms-like tyrosine kinase 3 ligand (FLT3L) is an important cytokine for the development of several hematopoietic populations, such as dendritic cells, B cells, regulatory T cells and natural killer cells. The FLT3L receptor (FLT3) is expressed on both myeloid and lymphoid progenitors and deletion of either the receptor or its ligand leads to defective developmental potential of hematopoietic progenitors. *In vivo* administration of FLT3L promotes expansion of progenitors with combined myeloid and lymphoid potential. To investigate further the role of FLT3L in hematopoietic development, we generated transgenic mice expressing high levels of human FLT3L. FLT3L transgenic mice displayed a dramatic expansion of dendritic and myeloid cells, leading to splenomegaly and blood leukocytosis. Bone marrow myeloid and lymphoid progenitors were significantly increased in numbers but retained their developmental potential. Furthermore, transgenic mice developed anemia together with a reduction in platelet numbers. FLT3L was shown to rapidly reduce erythrocyte numbers when injected into wild-type mice, indicating a direct negative role of the cytokine on erythropoiesis. We conclude that FLT3L acts on multipotent progenitors in an instructive way, inducing their development into myeloid/lymphoid lineages while suppressing their megakaryocyte/erythrocyte potential.

Introduction

The development of hematopoietic cells is a highly complex and tightly regulated process that in adults is initiated in the bone marrow (BM) from hematopoietic stem cells (HSC) and continues throughout life. All hematopoietic lineages are derived via intermediate multi-potent progenitors, which gradually lose their multi-potentiality and eventually become committed to one lineage. Several molecules are considered pivotal for the regulation of this process, including transcription factors, signaling proteins, adhesion molecules and cytokine receptors. Cytokines impart environmental signals into hematopoietic development and several of them have been identified as crucial for the generation of different hematopoietic lineages.(1) The role of cytokines in hematopoiesis is considered to be either instructive, by directly promoting differentiation of multi-potent progenitors into a specific lineage, or permissive, by selectively promoting the survival and/or proliferation of a particular lineage at the expense of others.(2) For most hematopoietic cytokines, their precise mode of action remains unknown.

As for other type III receptor tyrosine kinases, the Fms-like tyrosine kinase 3 (FLT3), or CD135, has an extracellular domain composed of 5 immunoglobulin-like domains and a tyrosine kinase motif in the cytoplasmic domain.(3-5) These features of FLT3 are shared with other hematopoietic cytokine receptors, such as Stem Cell Factor (SCF) receptor and platelet-derived growth factor (PDGF) receptor.(3) FLT3-Ligand (FLT3L) is the only known ligand for FLT3.(6) Both the soluble and the membrane bound form of FLT3L can bind FLT3, leading to receptor dimerization and subsequent activation of the tyrosine kinase domain.(7) Receptor activation initiates a signaling cascade involving proteins such as STAT5a, ERK1/2 and PI3K.(8)

FLT3 and its ligand have been the focus of considerable research due to their implication in leukemias, since several mutations in FLT3 have been identified in Acute Myeloid Leukemia (AML).(7) Amongst them, the most common is an internal tandem duplication of exon 14 of the *FLT3* gene (FLT3-ITD) that results in constitutive activation of the kinase domain.(9) This mutation is found in ~25% of AML and its presence constitutes a poor prognostic factor. FLT3-ITD confers growth factor-independent proliferation to leukemic cell lines and its expression in transgenic mice results in a fatal myeloproliferative syndrome.(10)

FLT3 is expressed by several hematopoietic cell populations.(11) Initially, it is expressed by non-self-renewing, short-term HSC.(12, 13) Several downstream progenitors with myeloid and/or lymphoid potential continue to express FLT3 whereas megakaryocyte/erythrocyte progenitors do not.(11, 14-18) With the exception of dendritic cells (DC), which retain FLT3 on their surface, FLT3 expression is down-regulated as cells undergo myeloid or lymphoid commitment. Deletion of either FLT3 or FLT3L resulted in defects in the developmental potential of myeloid/lymphoid progenitors underscoring the importance of FLT3 in their development.(19, 20) In addition, FLT3L deficient mice displayed reduced numbers of B cells, DC and Natural Killer (NK) cells(20), while FLT3L has been shown crucial in sustaining adult B lymphopoiesis.(21) Interestingly, ablation of the FLT3/FLT3L axis alone did not result in a complete block in the generation of any hematopoietic lineage, suggesting that FLT3L might exert its crucial role in hematopoiesis through interactions with other cytokines, such as Inteleukin-7 (IL-7) or SCF.(19, 22, 23)

To elucidate the specific action of FLT3L on hematopoiesis *in vivo*, administration of FLT3L has been carried out. Results obtained confirmed the important role of FLT3L in DC generation.(24) We have previously shown that apart from DC, FLT3L injection leads to transient expansion of a $FLT3^+$ progenitor population with lymphoid and myeloid potential.(25) In order to evaluate the role of FLT3L on the development of different hematopoietic lineages, we describe herein the effects of sustained over-expression of FLT3L in a transgenic mouse model. Our study confirms the positive role of FLTL in DC development and highlights the importance of this cytokine in the survival and expansion of lymphoid and myeloid progenitors. Furthermore, our data provide evidence for an instructive role of FLT3L in hematopoietic development.

Methods

Mice

All mice used herein were bred and maintained in our animal facility under pathogen free conditions and all animal experiments were performed within institutional guidelines (permission nos. 1887 and 1888). Immunizations to induce a T-dependent antibody response and FLT3L treatment of mice were carried out as previously described.(25)

Cell cultures

ST2, OP9 and OP9 stromal cells expressing the Notch ligand Delta-like 1 (OP9DL1) were maintained in IMDM supplemented with 5 x 10^{-5}M β-mercaptoethanol, 1mM glutamine, 0.03% w/v Primatone (Quest Naarden, The Netherlands), 100U/mL Penicillin, 100 μg/mL Streptomycin and 5% fetal bovine serum. Co-cultures of stromal cells with sorted progenitor cells were performed as previously described.(25) and Suppl. Materials and Methods.

Platelet counts

Blood was drawn from the tail vein of mice and incubated with 1% ammonium oxalate for 10 minutes at room temperature. Following incubation, live cells were counted in a Neubauer hemocytometer.

Immunofluorescence

Spleens were snap frozen and embedded in OCT-compound (Sakura, Zoetermeer, NL), and 5 μm sections were prepared. Sections were fixed in acetone for 10 min, air dried for 60 min and

subsequently stained with FITC-labeled anti-CD90, PE-labeled anti-IgM and APC-labeled anti-CD11c antibodies for 30 minutes.

Results

Splenomegaly and lymphadenopathy in FLT3L transgenic mice.

To investigate the effect of prolonged FLT3L over-expression, we generated mice expressing the human *FLT3L* gene under the control of the *β-actin* promoter (hereafter FLT3L-Tg mice). FLT3L levels in the blood were at the range of 500-1000 ng/ml, as assesed by ELISA using an anti-hFLT3L antibody developed in our laboratory (data not shown). FLT3L-Tg mice were viable, fertile with no apparent signs of disease until the age of 2-3 months, when many developed diarrhea and tail necrosis. Examination of internal organs revealed a striking increase in spleen size. Indeed, total spleen cellularity in 8-14 week old FLT3L-Tg mice was 451 ± 127 x 10^6 cells compared to 71 ± 12 x 10^6 cells in wild-type (WT) littermate controls, representing a 6.3-fold increase in total cell number (Figure 1A). Splenic architecture was disrupted with no clearly formed T-cell follicles and a dramatic increase in $CD11c^+$ dendritic cells (DC) (Figure 1B). Analysis of *axillary*, *brachial* and *inguinal* lymph node cellularity demonstrated an increase from 23 ± 2.6 x 10^6 cells in WT to 69.6 ± 3.5 x 10^6 cells in FLT3L-Tg mice (Figure 1A). Thymus cellularity and $CD4^+$/$CD8^+$ T cell subpopulations did not show any difference between WT and FLT3L-Tg mice (data not shown).

Increased FLT3L availability leads to alterations in the numbers of dendritic cells and B cells in the bone marrow.

Bone marrow cellularity (2 *femurs* and 2 *tibias*) was increased from 40 ± 7.7 x 10^6 cells in WT to 73.8 ± 14.5 x 10^6 cells in FLT3L-Tg mice (Figure 1A). Further analysis of BM myeloid and lymphoid populations revealed no significant change in the numbers of $GR1^+CD11b^+$

myeloid cells (data not shown). Staining for $CD19^+$ B cell progenitors revealed no change in the earliest B cell committed $CD19^+CD117^+$ preB1 population but a dramatic 12-fold (from 7 ± 2 x 10^6 in WT to 0.6 ± 0.3 x 10^6 cells in FLT3L-Tg) and 144-fold (from 8.3 ± 2.2 x 10^6 in WT to 0.06 ± 0.09 x 10^6 in FLT3L-Tg) decrease in $CD19^+CD117^-IgM^-$ preB2 and $CD19^+IgM^+$ B cells, respectively (Figure 1C). The apparent increase in total BM cellularity in FLT3L-Tg mice was partly due to a marked increase in DC populations. As shown in Figure 1D, $CD11c^+SiglecH^+$ plasmacytoid DC (pDC) showed a 41-fold increase (from 0.74 ± 0.15 x 10^6 in WT to 30 ± 8.8 x 10^6 in FLT3L-Tg), while $CD11c^+SiglecH^-$ conventional DC (cDC) numbers were increased 13-fold (from 0.3 ± 0.06 x 10^6 in WT to 2.5 ± 1.2 x 10^6 in FLT3L-Tg). Overall, analysis of the FLT3L-Tg BM lymphoid and myeloid populations revealed decreased numbers of B cell progenitors and a significant increase in DC numbers.

Diminished megakaryocyte/erythrocyte lineage differentiation, anemia and leukocytosis in FLT3L-Tg mice.

Alterations in BM erythroid progenitors in FLT3L-Tg mice were also investigated revealing a significant 5.4-fold decrease in $TER119^+$ erythroid progenitor numbers, from 2.7 ± 0.2 x 10^6 cells in WT to 0.5 ± 0.2 x 10^6 cells in FLT3L-Tg mice (Figure 2A). This decrease in $TER119^+$ erythroid progenitors prompted us to analyze the development of megakaryocyte/erythrocyte lineage in FLT3L-Tg mice. Hematocrit analysis showed that FLT3L-Tg mice manifested a significant decrease in hematocrit (from 46.4 ± 2 in WT to 34 ± 3 in transgenics) already at 8-10 weeks of age, which dropped even further to 21.6 ± 5.9 at 19-22 weeks of age (Figure 2B). Blood platelet counts also showed a significant drop, from 400 ± 89 x 10^8 cells/ml in WT to 207.5 ± 61 x 10^8 cells/ml in FLT3L-Tg mice (Figure 2C). Thus, megakaryocyte/erythrocyte lineage development seemed to be diminished, leading to anemia in FLT3L-Tg mice. Finally, blood smear preparations showed a marked increase in the numbers of leukocytes in FLT3L-Tg blood compared to WT (Figure 2D), which was confirmed by a quantitative analysis demonstrating a 27-fold increase in numbers of leukocytes in FLT3L-Tg blood (Suppl. Figure 1).

Expansion of myeloid and lymphoid populations in spleens of FLT3L-Tg mice.

Due to the observed splenomegaly we extended the analysis to mature hematopoietic populations in the spleen. Contrary to BM, the spleen of FLT3L-Tg mice displayed a significant (15-fold) increase of $GR1^+CD11b^+$ myeloid cells from 1.9 ± 0.7 x 10^6 cells in WT to 30.1 ± 7.6 x 10^6 cells in transgenics (Figure 3A). $NK1.1^+$ natural killer (NK) cells were also significantly increased from 2.7 ± 0.8 x 10^6 in WT to 23 ± 6.3 x 10^6 (Figure 3A). A detailed analysis of DC subsets in the spleen of FLT3L-Tg mice showed a dramatic increase in DC numbers, reaching 368-fold for $CD11c^+SiglecH^+$ pDC (95.8 ± 26 x 10^6 versus 0.26 ± 0.06 x 10^6 in WT), 208-fold for $CD11c^+CD11b^+$ cDC (91.7 ± 25 x 10^6 versus 0.44 ± 0.29 x 10^6 in WT) and 161-fold for $CD11c^+CD8\alpha^+$ cDC (21.6 ± 7.2 x 10^6 versus 0.13 ± 0.05 x 10^6 in WT) (Figures 3 B, D). These expanded DC populations in our FLT3L-Tg mice were shown to be functional (Suppl. Figure 2). We conclude from this data that the massive expansion of myeloid, NK and, mainly, DC populations accounts for the splenomegaly observed in FLT3L-Tg mice.

The apparent reduction in BM B lymphopoiesis was not reflected in a reduction of $CD19^+IgM^+$ mature B cell numbers in the spleens of FLT3L-Tg mice (Figure 3C). In contrast, despite the similarity in thymus cellularity, there was nevertheless a 3.2-fold increase (from 11.5 ± 4.3 x 10^6 in WT to 37.4 ± 19 x 10^6 cells in FLT3L-Tg) in splenic $CD4^+$ T cells (Figure 3C). The increase in $CD8^+$ T cell number was smaller (2.4-fold); from 7.8 ± 2.8 x 10^6 cells in WT to 18.9 ± 8.4 x 10^6 cells in FLT3L-Tg mice. Using intracellular FACS staining, a 7.4-fold increase in $Foxp3^+CD4^+$ regulatory T cells (Tregs) (from 1.36 ± 0.2 x 10^6 in WT to 10.14 ± 1.3 x 10^6 in FLT3L-Tg), was also detected in the spleen of FLT3L-Tg mice (Figure 3C), as shown previously by *in vivo* FLT3L injection.(26)

The abnormal splenic architecture and alterations in some T cell numbers motivated us to test the quality of immune response in FLT3L-Tg. Thus, transgenic and WT control littermates were immunized with NIP protein and the levels of serum anti-NIP IgG antibodies were quantified 13 days later by ELISA. Anti-NIP IgG responses were somewhat weaker in FLT3L-Tg mice compared to WT, even though a significant increase in IgG titers was still observed after immunization (Suppl. Figure 3). Interestingly, pre-immune serum of FLT3L-Tg mice contained slightly higher IgG immunoglobulin levels than that of controls.

Expansion of hematopoietic progenitors in the BM of FLT3L-Tg mice.

Increased availability of FLT3-ligand was previously shown to expand DC populations and increase numbers of an $FLT3^+$ progenitor population named Early Progenitor with Lymphoid and Myeloid potential (EPLM).(25) In addition to EPLM, other lymphoid and myeloid progenitor populations such as LMPP (Lymphoid-primed Multipotent Progenitor Population),(27) CLP (Common Lymphoid Progenitors)(28) and CMP (Common Myeloid Progenitors)(29) are known to be $FLT3^+$. To assess the potential effects of constitutive FLT3L over-expression on these progenitor populations, we analyzed them in the BM of FLT3L-Tg mice. Most of the CLP, EPLM and a fraction of myeloid restricted $CD117^+CD115^+Sca1^-$ cells were indeed $FLT3^+$, but FLT3 expression was downregulated in FLT3L-Tg mice, presumably due to continuous engagement and internalisation of the receptor (Suppl. Figure 4). Therefore, we have not used FLT3 as a marker in our analysis. As shown previously,(25) upon increased FLT3L availability, there was a 14-fold increase in EPLM ($CD117^+B220^{low}CD19/NK1.1^-$) numbers from $0.2 \pm 0.1 \times 10^6$ cells in WT to $2.8 \pm 0.9 \times 10^6$ cells in FLT3L-Tg mice (Figures 4 A, B). Increases in CLP ($CD117^+Sca1^+CD127^+$) progenitors were even higher with a 75-fold increase from $0.04 \pm 0.01 \times 10^6$ cells in WT to $3.2 \pm 1.3 \times 10^6$ cells in FLT3L-Tg mice. While staining for CLP, we noted a $CD117^-CD127^+$ population that was increased in FLT3L-Tg mice. This population could not represent B cell progenitors, since they are reduced in FLT3L-Tg mice. Indeed, further analysis revealed these cells were $CD11c^+SiglecH^+$ pDC, which had expanded 41-fold in FLT3L-Tg mice BM and were all $CD127^+$ (Suppl. Figure 5). $CD117^+CD115^+Sca1^-$ myeloid progenitors were also found to be significantly increased in FLT3L-Tg mice (from $0.4 \pm 0.1 \times 10^6$ cells in WT to $5.1 \pm 1.4 \times 10^6$ in FLT3L-Tg). The $Lineage^-Sca1^+CD117^+$ (LSK) fraction, which is to a large extent $FLT3^+$ (Suppl. Figure 4), was also significantly increased in FLT3L-Tg mice (Figures 4 C, D). LSK cells contain $FLT3^-$ LT-HSC and $FLT3^+$ ST-HSC and multi-potent progenitors.(12) Due to our inability to stain for FLT3 in FLT3L-Tg mice, we stained LSK cells with CD150 instead, which marks a population highly enriched in HSC.(30) Even though percentage wise they were reduced, no significant change was detected in absolute numbers of $CD150^+$LSK cells in FLT3L-Tg mice (Suppl. Figure 6).

The anemia and reduction in TER119$^+$ erythroid progenitors and platelets prompted us to investigate the earliest identified megakaryocyte/erythroid progenitors. Staining the CD117$^+$Sca1$^-$CD127$^-$ fraction of BM with CD34 and CD16 allows the identification of Common Myeloid Progenitors (CMP), as well as progenitors with restricted Granulocyte-Macrophage (GMP) and Megakaryocyte-Erythrocyte (MEP) potential.(29, 31) We found a dramatic 9.7-fold decrease in numbers of MEPs in FLT3L-Tg mice compared to WT, as well as a significant 5.2-fold increase in GMPs (Figures 4 C, D). These results indicate that increased FLT3L levels skew development towards the myeloid/lymphoid and away from the megakaryocyte/erythroid pathway and that this skewing occurs already at the level of FLT3$^+$ multipotent progenitors.

Next, we determined whether the expansion of lymphoid and myeloid progenitors in FLT3L-Tg mice was accompanied by a change in their developmental potential. Therefore, EPLM, CLP and CD117$^+$CD115$^+$Sca1$^-$ cells were sorted from WT or FLT3L-Tg mice and plated in 96-well plates under differentiation conditions promoting myeloid (ST2 stromal cells), B-cell (OP9 stromal cells plus IL-7) and T-cell (OP9DL1 stromal cells plus IL-7) development. As shown in Table 1, our *in vitro* differentiation analysis showed no significant change in the developmental potential of the analyzed progenitor populations. To assess the *in vivo* potential of progenitors, CLP were sorted from FLT3L-Tg mice and transplanted into *Rag2*$^{-/-}\gamma c^{-/-}$ recipients. After 3 weeks, CLP from FTL3L-Tg demonstrated robust B and T cell reconstitution potential (Suppl. Figure 7). Transplantation of CD45.2 FLT3L-Tg EPLM into CD45.1 congenic mice revealed their combined lymphoid and myeloid potential (Suppl. Figure 8), as shown before for WT EPLM.(32) Finally, transplantation of FLT3L-Tg LSK in a similar setting demonstrated their potent myeloid reconstitution potential but a reduced erythroid potential compared to WT LSK (Suppl. Figure 9). We conclude that the lymphoid and myeloid potential of FLT3L-Tg progenitors is retained, while their erythroid potential is reduced.

Kinetics of hematopoietic population changes suggest an instructive role of FLT3L in hematopoiesis.

The observed alterations in hematopoietic populations in FLT3L-Tg mice could be the consequence of a direct, “instructive”, action of FLT3L on multi-potent progenitors, actively

guiding them to acquire a particular cell fate at the expense of other options ("instructive" model). Alternatively, over-expression of FLT3L could result in a vast expansion of $FLT3^+$ cells which could leave little space and/or recourses for non-expanding cells, thus leading to a reduction in their numbers ("space" model). In a system with sustained elevated levels of FLT3L it would be difficult to distinguish between the two possibilities. To acquire data supportive of either model, we injected WT mice with recombinant FLT3L and monitored kinetic changes in numbers of different hematopoietic lineages. As shown in Figures 5 A and B, and in accordance with the anemic phenotype of FLT3L-Tg mice, the percent nucleated BM MEP and $TER119^+$ erythroid progenitors showed a reduction by 3 days post FLT3L injection, which was already significant in the case of MEP. Considering the turnover of $TER119^+$ erythroid progenitors, the speed by which increased FLT3L availability leads to MEP and TER119+ progenitor reduction would argue for an instructive, negative, role of FLT3L in their generation.

In addition, we quantified the percentages of other hematopoietic cells whose numbers were significantly altered in FLT3L-Tg mice, namely pDC and $CD19^+$ B cell progenitors. Plasmacytoid DC seemed to also increase, demonstrating a 2.6-fold increase at 5 days after FLT3L injection and reaching a 5.8-fold increase at day 7 (Figure 5B). This would be consistent with a role of FLT3L in expansion of these $FLT3^+$ cells, shown previously.(24, 25) $CD19^+CD117^+$ preB1 cells showed little reduction; rather an up to 2-fold increase in their percentage was observed at day 7 (Figure 5C). On the contrary, and in accordance with our FTL3L-Tg analysis, both $CD19^+CD117^-IgM^-$ preB2 and $CD19^+IgM^+$ B cells were reduced after FLT3L injection, becoming significant after 5 days (Figure 5 D-E). Overall, our FLT3L injection data would point towards an instructive role of FLT3L in development of certain hematopoietic lineages.

Discussion

Several lines of evidence point towards an important role of FLT3/FLT3L in hematopoiesis. The relatively mild phenotype of both FLT3 and FLT3L knock-out mice would

suggest that this cytokine exerts its role mainly in concert with other hematopoietic cytokines, such as Stem Cell Factor (SCF) or Interleukin-7 (IL-7).(19, 22) Administration of FLT3L to adult mice *in vivo* has been used as a means of elucidating its exact role in regulating hematopoiesis.(24, 25) In the present study we report for the first time a detailed *in vivo* analysis of the effect of elevated and sustained transgenic expression of FLT3L in different hematopoietic lineages. Our results suggest an instructive role of FLT3L in hematopoietic development.

Analysis of FLT3L-Tg mice showed significant alterations in several hematopoietic lineages. The population with the highest increase was DC. Both in BM and spleen, all DC populations displayed a dramatic expansion ranging from 7- to 368-fold compared to WT mice. Previously, FLT3L was shown to be a crucial factor for the *in vitro* generation of DC,(33) whereas *in vivo,* absence of FLT3L resulted in a marked decrease in DC numbers.(20) Increases in DC were also noted in studies where either FLT3L was administered *in vivo*(24, 25) or when FLT3L was conditionally over-expressed by transgenesis.(34) The importance of FLT3L in DC generation has been shown for cDC and pDC, both of which are $FLT3^+$ populations. Our results are in accordance with these observations. Particularly striking was the elevated numbers of pDC, the population most increased in our transgenic mice. Plasmacytoid DC are considered an important part of anti-viral immunity, mainly through their production of IFN-α.(35) The dramatic increase of IFN-α producing pDC upon sustained over-expression of FLT3L suggests a potential therapeutic use of this cytokine to combat chronic viral infections. Furthermore, our transgenic system constitutes a source for the *ex vivo* isolation of vast numbers of functional DC populations.

A significant reduction in $CD19^+CD117^-IgM^-$ preB2 and $CD19^+IgM^+$ B cell populations was noted in the BM of FLT3L-Tg mice. This phenotype was somewhat surprising, considering the decreased numbers of B cell progenitors in mice deficient in functional FLT3 or FLT3L, results that had suggested a positive role of FLT3L in B cell development.(19, 20, 36) Furthermore, coincident with CD19 expression and due to repression by PAX5, FLT3 is down-regulated in B cell progenitors,(37) thereby excluding the possibility that FLT3L is necessary for the survival of $CD19^+$ cells. Moreover, there was no evidence for reduced B cell potential among CLP and EPLM from FLT3L-Tg mice in which there were normal numbers of the earliest committed $CD19^+CD117^+$ preB1 cells. We consider decreased IL-7 availability the most likely

explanation for this apparent reduction in BM CD19$^+$ cells. There was a dramatic increase in IL7Rα$^+$ (CD127$^+$) lymphoid progenitors, such as CLP (76-fold) and EPLM (14-fold) in FLT3L-Tg mice. Furthermore, pDC, which we found to be IL7Rα$^+$, were also increased (41-fold). Hence, there is an enormous expansion of IL7Rα$^+$ cells in FLT3L-Tg mouse BM which, by absorbing IL-7, could lead to reduced levels of available IL-7 necessary for CD19$^+$ cell survival and/or proliferation. As a population, preB2 cells are particularly sensitive to IL-7 availability, while the reduced numbers of IgM$^+$ B cells might reflect the reduced input from the preB2 stage. To test this hypothesis we injected FLT3L-Tg mice with IL-7/anti-IL-7 complexes(38) and were able to increase preB2 and B cell percentages almost to WT levels (Suppl. Figure 10). Despite the reduction in BM preB2 and B cells, FLT3L-Tg BM output seemed sufficient to reconstitute the splenic B cell compartment. Overall, we believe that the diminished numbers of BM CD19$^+$ B cell progenitors in FLT3L-Tg mice is a secondary effect, caused by the decreased availability of IL-7 rather than a negative role of FLT3L in B cell development and survival.

In the BM of FLT3L-Tg, numbers of FLT3$^+$ lymphoid and myeloid progenitors were dramatically increased. In a previous study, EPLM were also increased following administration of FLT3L *in vivo*, while their B lineage potential in limiting dilution assays was decreased.(25) Despite their expansion in our FLT3L-Tg mice, the B, T and myeloid lineage potential of EPLM was unaltered. This apparent difference to the *in vivo* administration data could be due to the sustained elevated levels of FLT3L in FLT3L-Tg mice. This dramatic expansion without any apparent alteration in the developmental potential of lympho-myeloid progenitors analyzed would indicate a role of FLT3/FLT3L signaling in their survival and/or proliferation, rather than in instructing them towards a particular downstream lineage fate. In addition, elevated FLT3L levels could enhance the generation of these FLT3$^+$ progenitors from HSC. Given the very small numbers of CMP, CLP and EPLM in a WT BM, our FLT3L-Tg mice provide an excellent mouse model for the isolation of these progenitors in large numbers for further *in vitro*, *in vivo* or molecular biology analyses.

Strikingly, FLT3L-Tg mice became severely anemic a few months after birth. Both platelets and erythrocytes were diminished in these mice, suggesting a defect in the generation of the megakaryocyte/erythrocyte lineage. One explanation for this phenotype could be that the dramatic expansion of several other cell types in FLT3L-Tg mice would lead to a reduction of

FLT3$^-$ megakaryocyte/erythrocyte progenitors due to competition for space and/or resources. Nevertheless, the dramatic reduction in MEP in FLT3L-Tg mice and their rapid decrease following FLT3L treatment argues for a defect in their development rather than having no space to expand. This developmental defect would in turn suggest that erythrocytes originate from an FLT3L-responsive population that can be induced by FLT3 signaling to develop into lympho-myeloid lineages at the expense of the erythroid lineage. Based on our data, we propose that FLT3$^+$ non self-renewing HSC that receive sufficient FLT3L signal differentiate to lympho-myeloid progenitors, while HSC that do not activate FLT3 signaling, due to either low FLT3 expression and/or low FLT3L levels in their microenvironment, develop into megakaryocyte-erythrocyte progenitors (Figure 6). Increased FLT3L availability would result in very few, if any, FLT3$^+$ HSC not receiving an adequate FLT3L signal, thus leading to decreased megakaryocyte/erythrocyte developmental input and increased lymphoid-myeloid progenitor compartment, as is the case in our FLT3L-Tg mice. Our data strengthen the suggested importance of FLT3 signaling in promoting lympho-myeloid versus megakaryocyte-erythroid lineage development(27) and provide evidence for an instructive role of FLT3L in this process. Furthermore, they are in accordance with recent data demonstrating that platelets and erythrocytes originate from *Flt3*-expressing progenitors.(39-41)

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Tables

Table 1.

***In vitro* developmental potential of CD117$^+$CD115$^+$Sca1$^-$, CLP and EPLM populations from wild type and FLT3L-Tg mice.**

		B cell potential	T cell potential	Myeloid potential
CD117$^+$CD115$^+$ Sca1$^-$	WT	<1 in 120	1 in 76	1 in 3
	TG	<1 in 120	1 in 42	1 in 2
CLP	WT	1 in 10	1 in 15	1 in 12
	TG	1 in 5	1 in 3	1 in 5
EPLM	WT	1 in 5	1 in 10	1 in 16
	TG	1 in 10	1 in 3	1 in 6

Frequencies of progenitors with B cell, T cell and myeloid cell potential as assessed by *in vitro* limiting dilution analysis. One representative experiment is shown out of 3-6 for the different populations. CLP: Common Lymphoid Progenitor, EPLM: Early Progenitors with Lymphoid and Myeloid potential, WT: wild-type, TG: FLT3L transgenics.

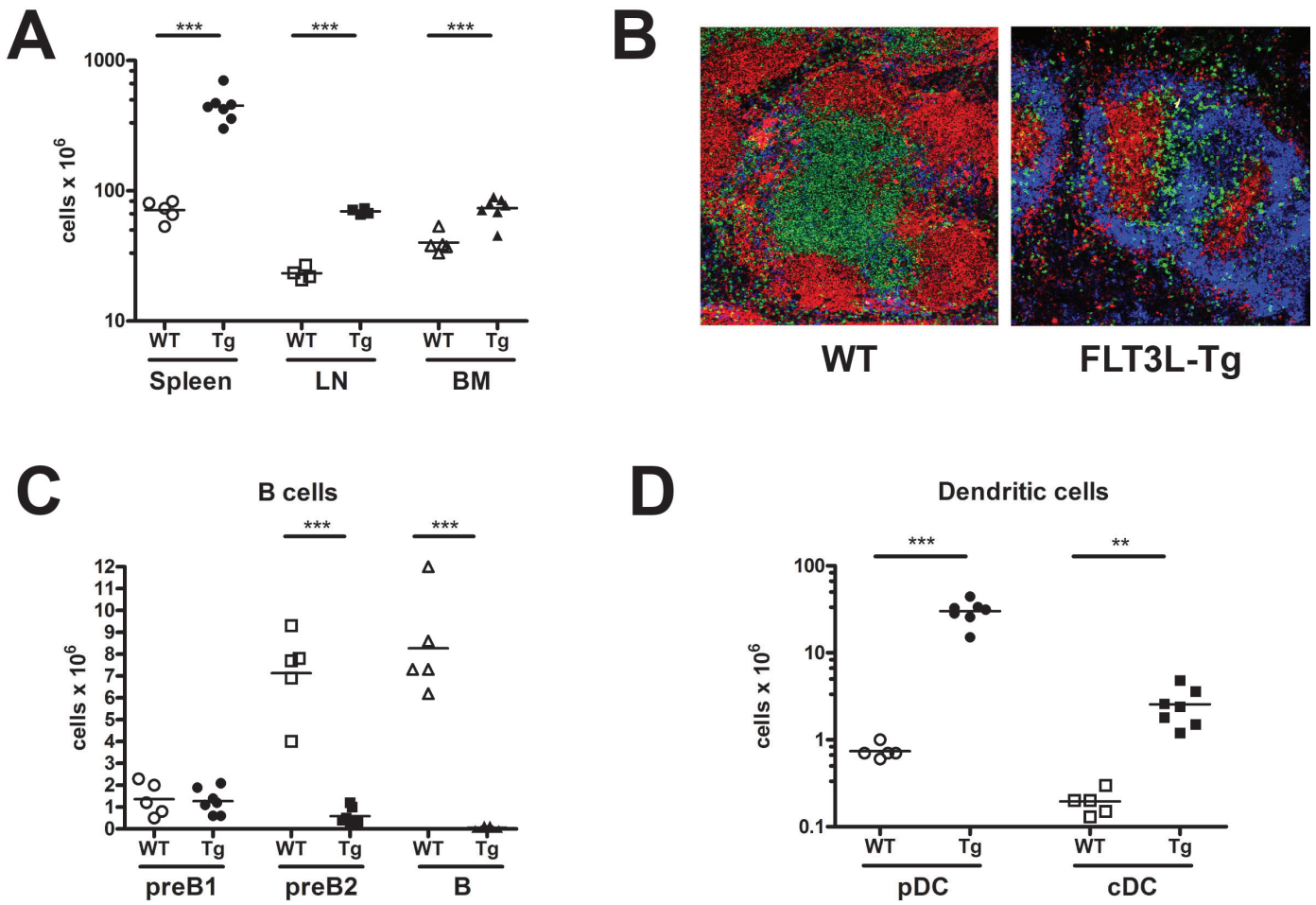


Figure1. *Splenomegaly, lymphadenopathy and disrupted spleen architecture in FLT3L-Tg mice*.
A. Total cellularity in spleen, lymph nodes (axillary, brachial and inguinal) and bone marrow (2 femurs and 2 tibias) of 8-14 week old wild type (WT - white symbols) and FLT3L-Tg (black symbols) mice (5-7 mice per group). ***: P<0.0001. B. Immunofluorescence of spleen sections from 8-14 week old WT and FLT3L-Tg mice stained for B cells (anti-IgM, red), T cells (anti-CD90, green) and dendritic cells (anti-CD11c, blue). C. Numbers of CD19+CD117+ (preB1), CD19+CD117-IgM- (preB2) and CD19+IgM+ (B) B cells in WT (white symbols) and FLT3L-Tg (black symbols) mice (5-7 mice per group). ***: P<0.0001. D. CD11c+SiglecH+ plasmacytoid (pDC) and CD11c+SiglecH- conventional (cDC) dendritic cell numbers in WT (white symbols) and FLT3L-Tg (black symbols) mice (5-7 mice per group). ***: P<0.0001, **: P=0.0022.

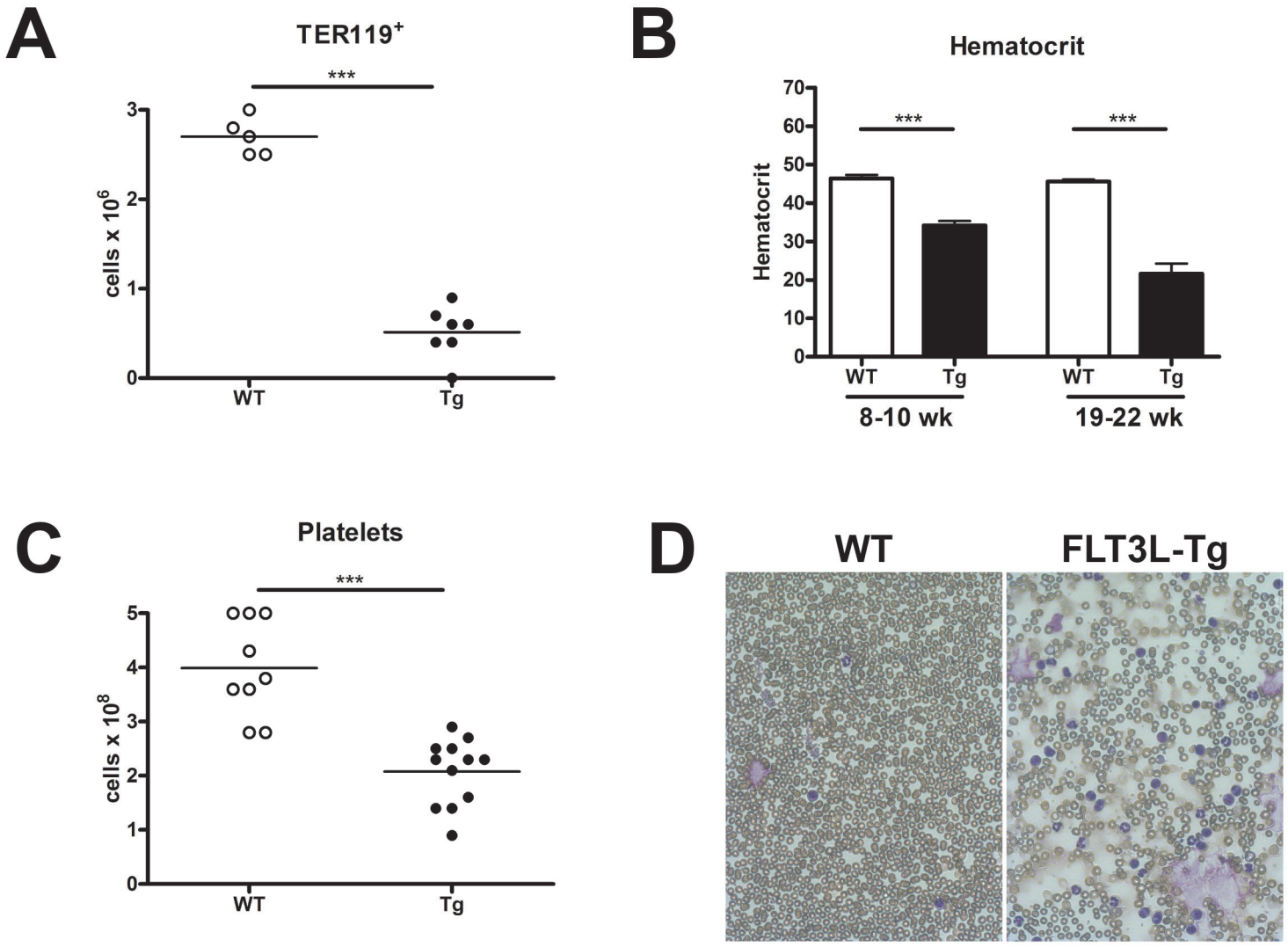


Figure 2. *Diminished megakaryocyte/erythrocyte lineage development, anemia and leukocytosis in FLT3L-Tg mice*.

A. Numbers of TER119+ erythroid progenitors in the bone marrow (2 femurs and 2 tibias) of 8-14 week old WT (white circles) and FLT3L-Tg (black circles) mice (5-7 mice per group). ***: P<0.0001. B. Hematocrit levels in WT (white bars) and FLT3L-Tg (black bars) mice (5-7 mice per group). ***: P<0.0001. C. Numbers of platelets in the blood of WT (white circles) and FLT3L-Tg (black circles) mice (9-12 mice per group). ***: P<0.0001. D. Hematoxylin-eosin staining of blood smears from WT and FLT3L-Tg mice (40x magnification).

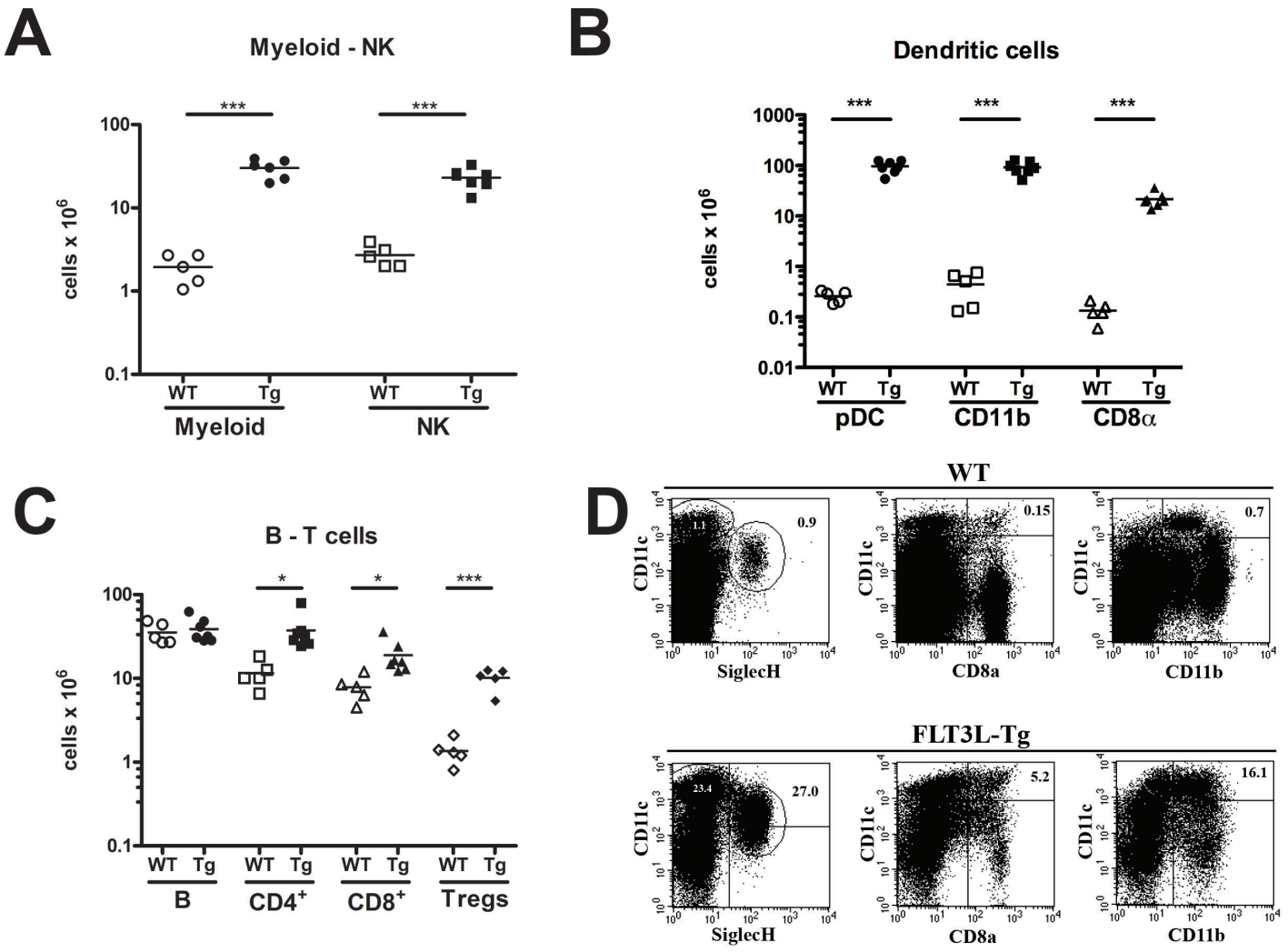


Figure 3. *Alterations in myeloid, lymphoid and dendritic cell populations in the spleens of FLT3L-Tg mice*. A. Numbers of CD11b+GR1+ myeloid and NK1.1+ NK cells in spleens of 8-14 week old WT (white symbols) and FLT3L-Tg (black symbols) mice (5 or 6 mice per group). ***: P<0.0001. B. Numbers of CD11c+SiglecH+ plasmacytoid DC (pDC), CD11c+CD11b+ conventional DC (CD11b) and CD11c+CD8α+ conventional DC (CD8α) in spleens of WT (white symbols) and FLT3L-Tg (black symbols) mice (5 or 6 mice per group). ***: P<0.0001. C. Numbers of CD19+IgM+ B cells, CD4+ T cells, CD8+ T cells and Foxp3+CD4+ regulatory T cells (Tregs) in the spleens of WT (white symbols) and FLT3L-Tg (black symbols) mice (5 or 6 mice per group). *(CD4): P=0.149, *(CD8): P=0.0192, ***: P<0.0001. D. FACS plots showing the gating strategy used for staining the DC subpopulations included in B.

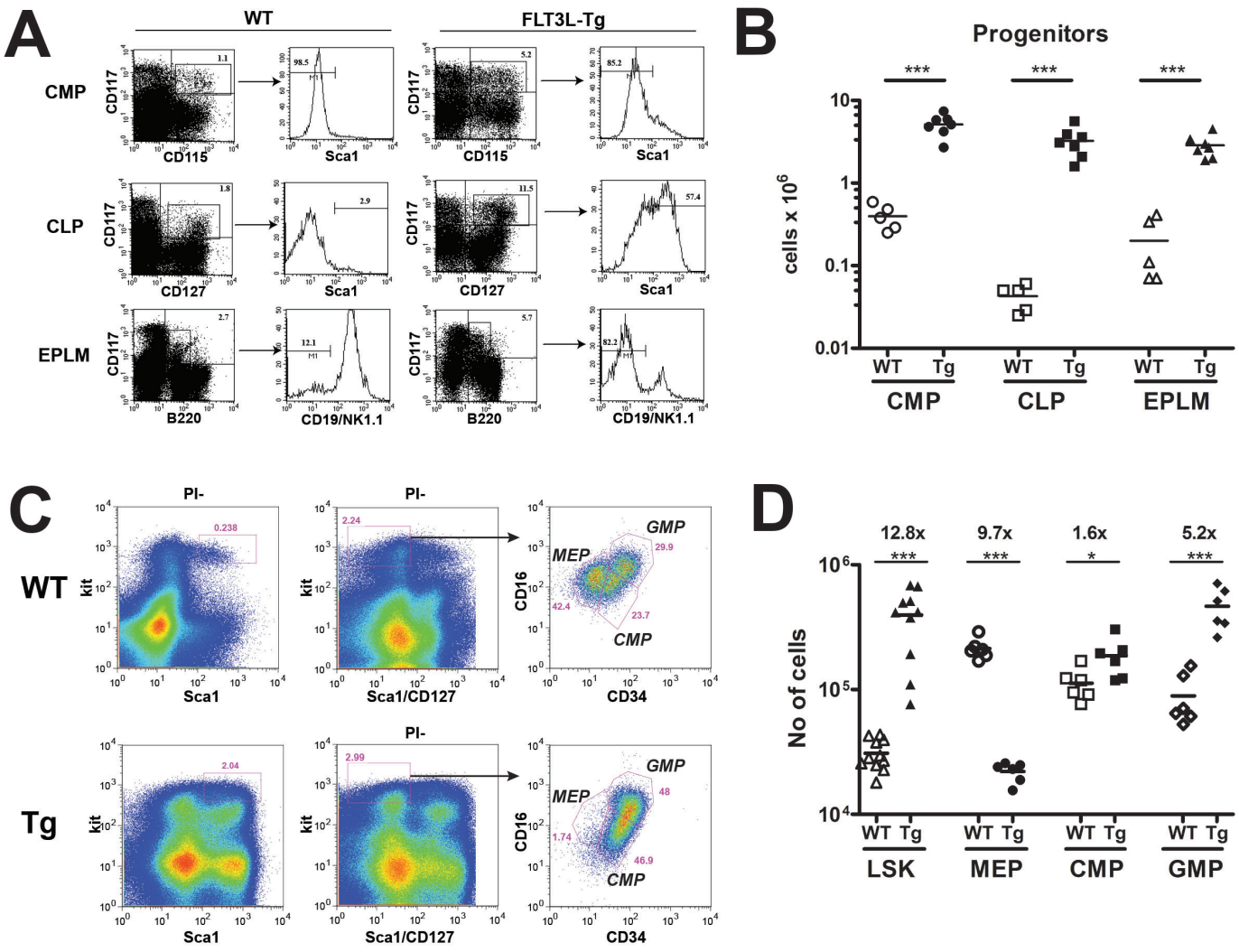


Figure 4. *Hematopoietic progenitor populations in FLT3L-Tg mice*.
Figure depicts numbers (B and D) and FACS stainings (A and C) of hematopoietic progenitor populations in the bone marrow (2 femurs and 2 tibias) of 8-14 week old mice. A. Representative FACS plots demonstrating the gating strategy used for staining CD117+CD115+Sca1- (upper panel), CLP (middle panel) and EPLM (lower panel) in WT and FLT3L-Tg bone marrows. B. Total numbers of CD117+CD115+Sca1-, CLP and EPLM in the bone marrow of WT (white symbols) and FLT3L-Tg (black symbols) mice (5-7 mice per group). ***: P<0.0001. C. Representative FACS plots demonstrating the gating strategy used for staining LSK (left panel) and MEP, CMP, GMP (centre and right panel) in WT and FLT3L-Tg bone marrows. D. Total numbers of LSK, MEP, CMP and GMP in the bone marrow of WT (white symbols) and FLT3L-Tg (black symbols) mice (MEP, CMP, GMP: 6 mice per group; LSK: 10 mice per group).

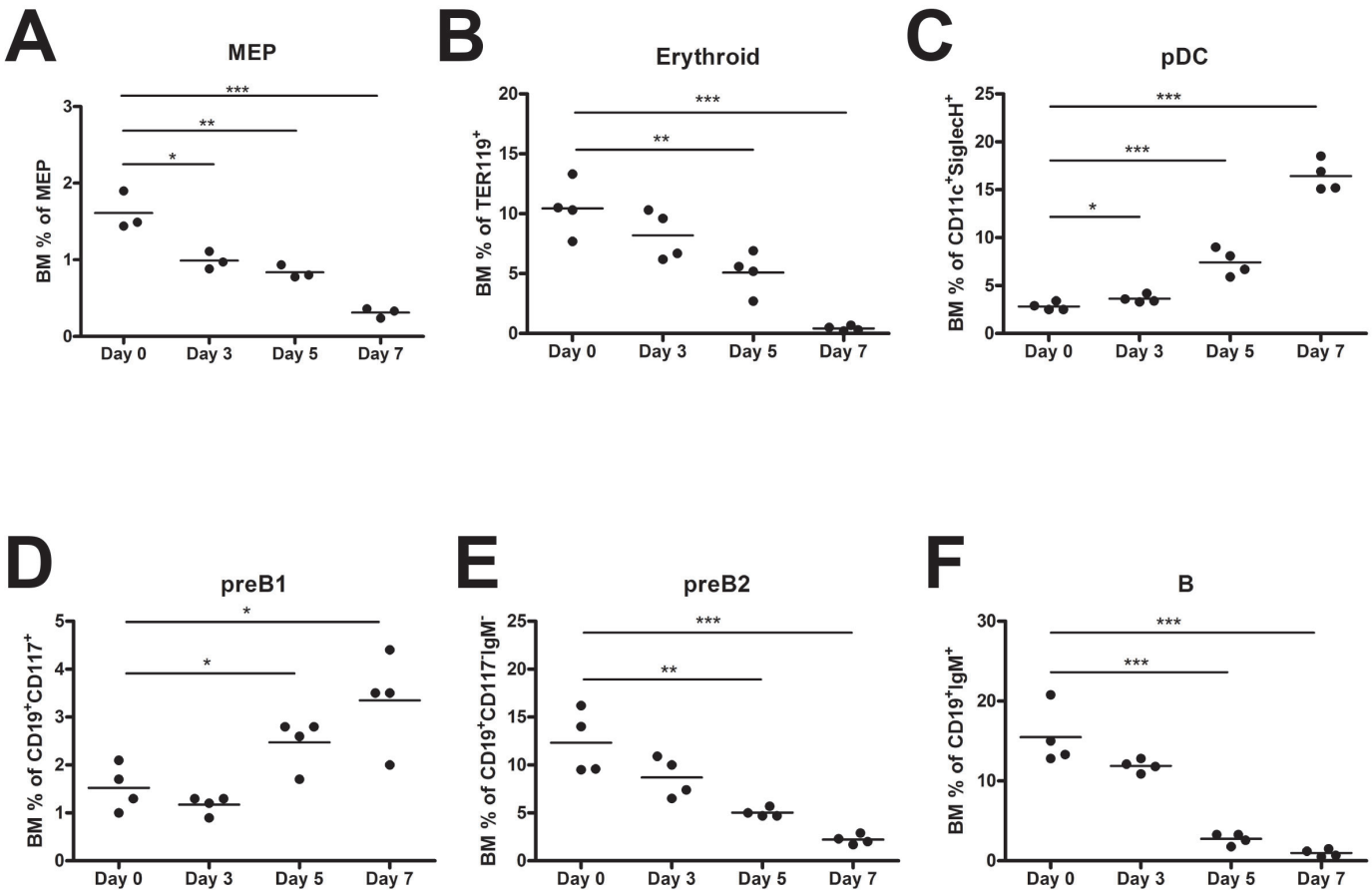


Figure 5. *Kinetics of changes in percentages of hematopoietic populations following FLT3L injections into wild type mice*.

Figure shows percentages of hematopoietic cells in the BM of WT mice injected with 10 μg of recombinant FLT3L 0, 3, 5 and 7 days after injection. A. Percentages of MEP. B. Percentages of nucleated TER119+ bone marrow cells. **: P=0.01, ***: P=0.0001. C. Percentages of CD11c+SiglecH+ plasmacytoid dendritic cells. *: P=0.0345, **: P=0.0007, ***: P<0.0001. D. Percentages of CD19+CD117+ preB1 cells. *(Day 5): P=0.0368, *(Day 7): P=0.0163. E. Percentages of CD19+CD117-IgM- preB2 cells. **: P=0.0049, ***: P=0.0001. F. Percentages of CD19+IgM+ B cells. ***(Day 5): P=0.0005, ***(Day 7): P=0.0002.

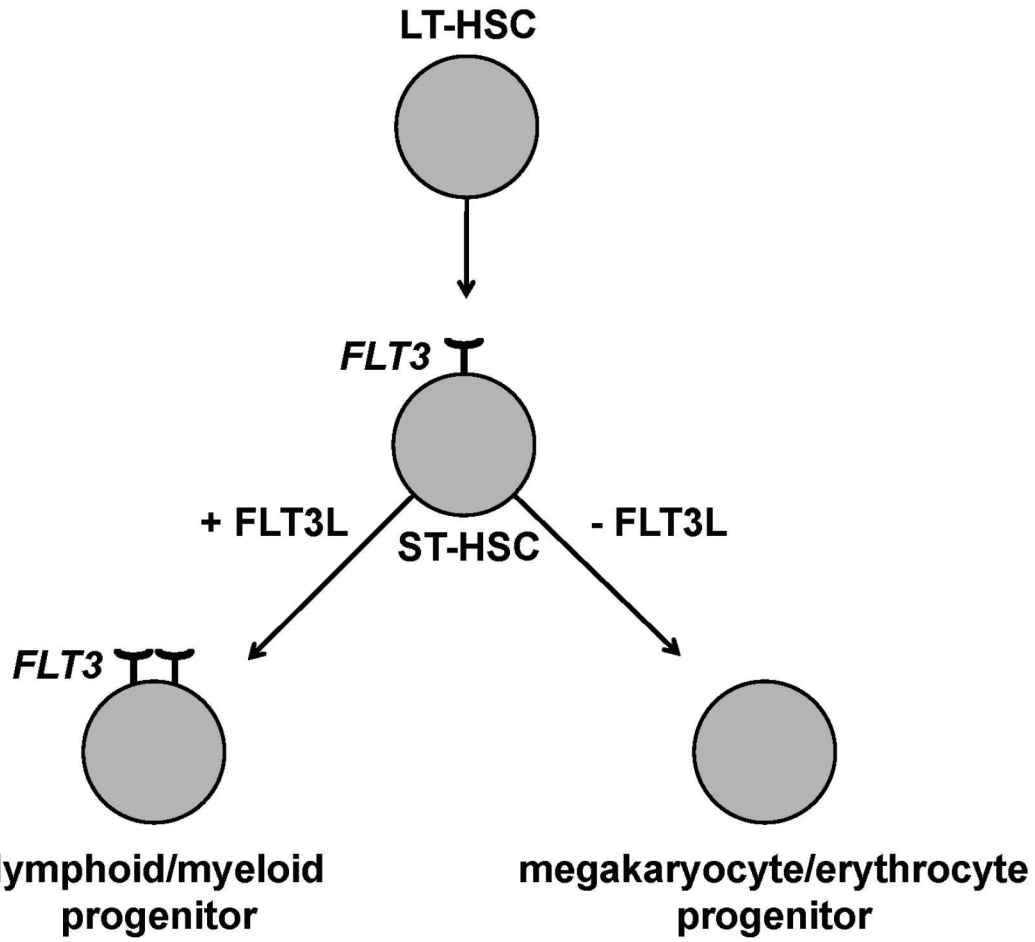
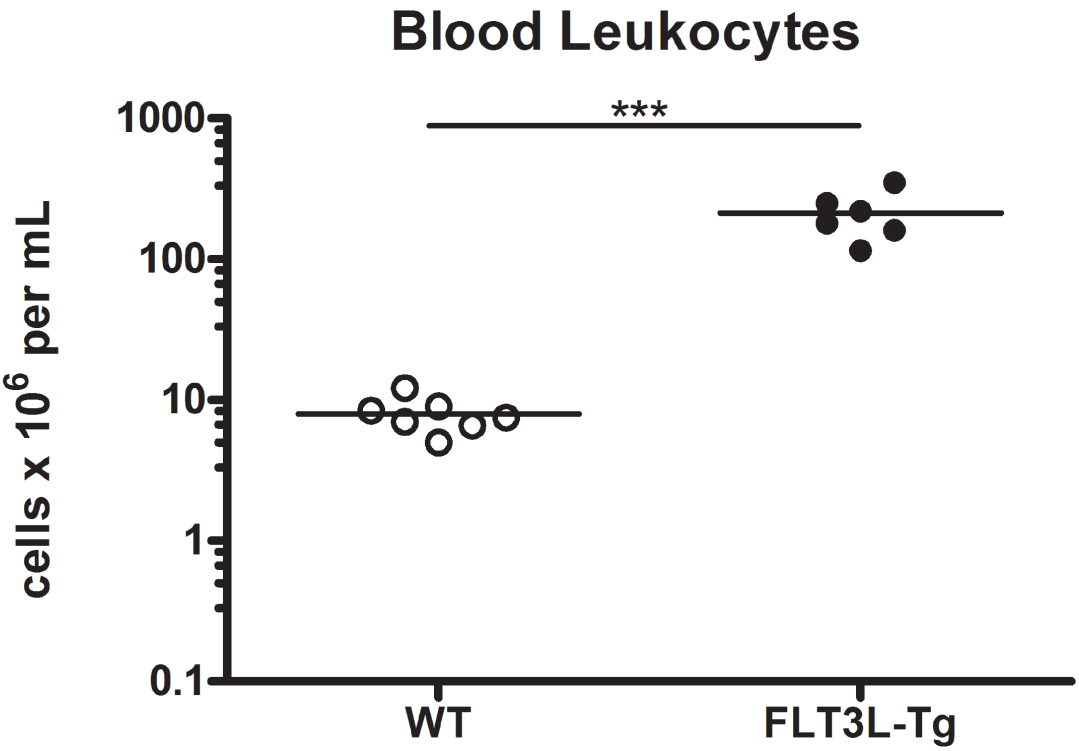


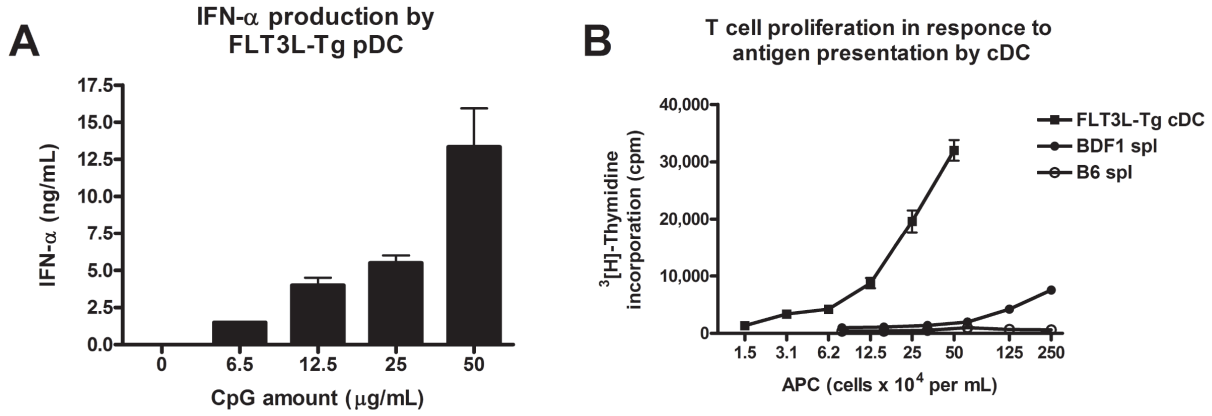
Figure 6. *Proposed model for the instructive action of FLT3L in determining lymphoid/myeloid versus megakaryocyte/erythrocyte lineage development.*



Supplementary Figure 1

Leukocytosis in FLT3L-Tg blood.

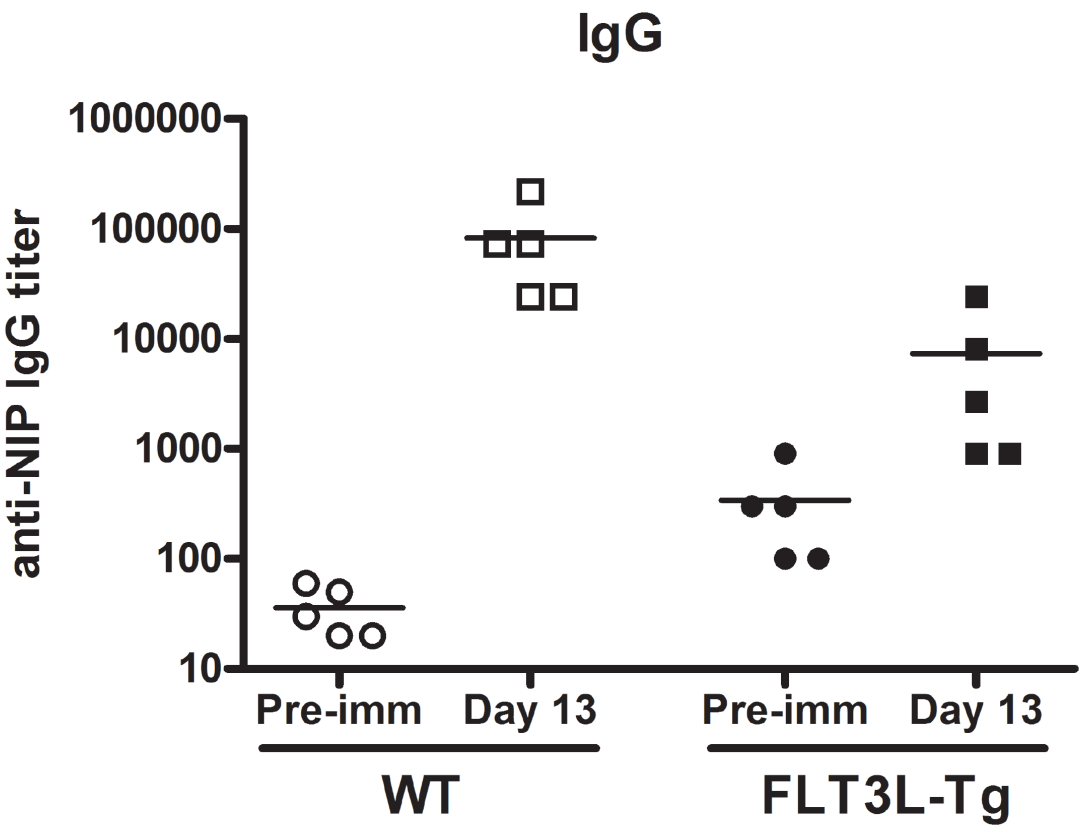
Blood was drawn from the tails of 8-14 week old WT (white circles) and FLT3L-Tg (black circles) mice. Following 5 minute incubation with NH_4Cl to lyse erythrocytes, live white blood cells were counted in a Neubauer hemocytometer. ***: P<0.0001



Supplementary Figure 2

Functional analysis of FLT3L-Tg DC subsets

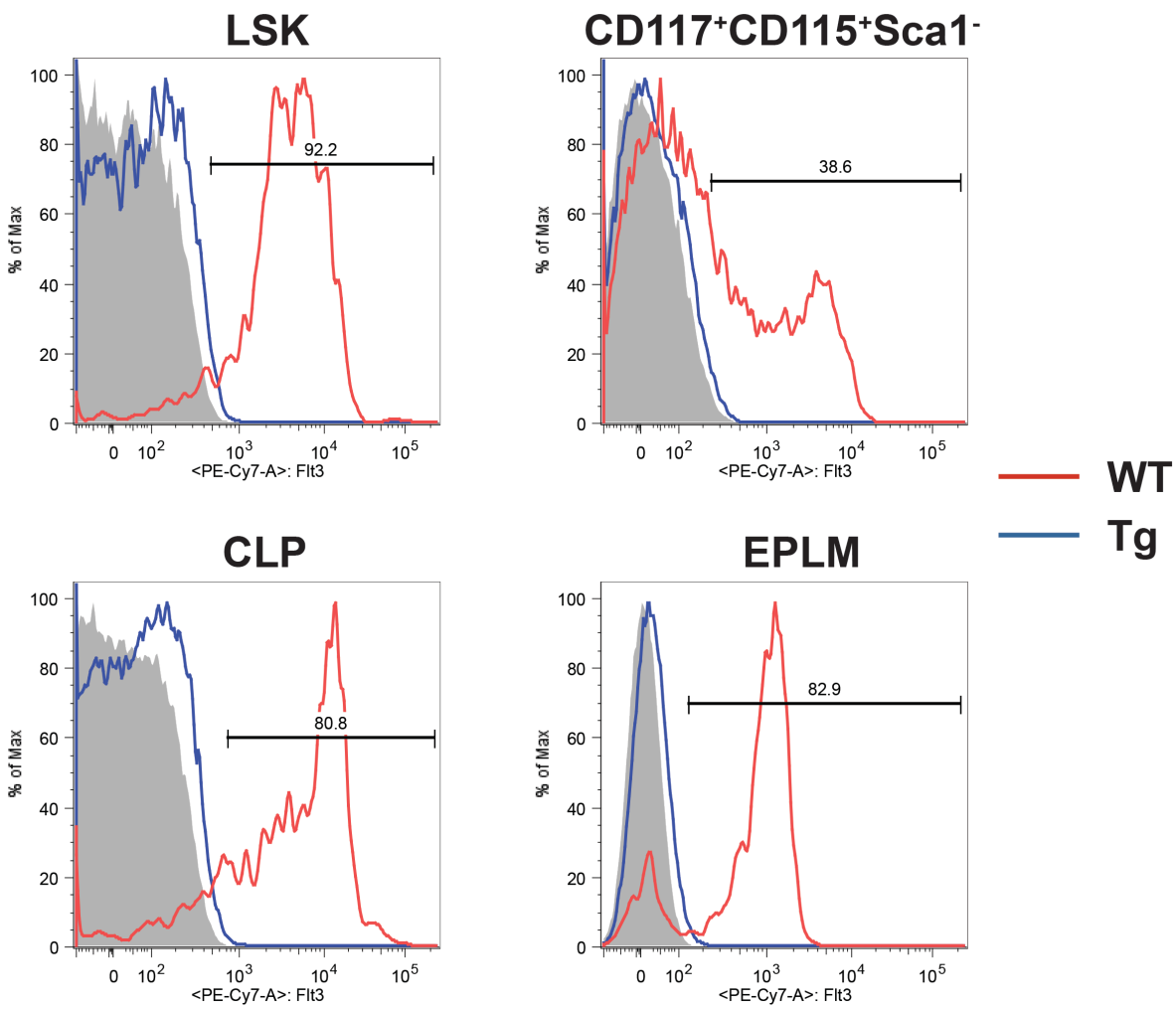
A. $CD11c^+SiglecH^+$ pDC were sorted from FLT3L-Tg spleens and stimulated with increasing amounts of CpG for 24 hours. Following stimulation, supernatants were collected and the amount of IFN-α produced was quantified using the Mouse IFN-alpha Platinum ELISA (eBioscience) according to manufacturer's instructions. B. $CD11c^+CD11b^+$ cDC were sorted from spleens of FLT3L-Tg mice [(C57BL/6 x DBA/2)F1, herein named BDF1] and incubated with lymph node (LN) cells from WT C57BL/6 mice, as a source of T cells, for 5 days. Following incubation, proliferation of LN cells was quantified by measuring ^{3}[H]-Thymidine incorporation. As a negative control, spleen cells from WT C57BL/6 were incubated with WT C57BL/6 LN cells (B6), while incubation of WT BDF1 spleen cells with WT C57BL/6 LN cells was used as a positive control for T cell proliferation (BDF1).



Supplementary Figure 3

IgG anti-NIP titers of FLT3L-Tg sera after immunization.

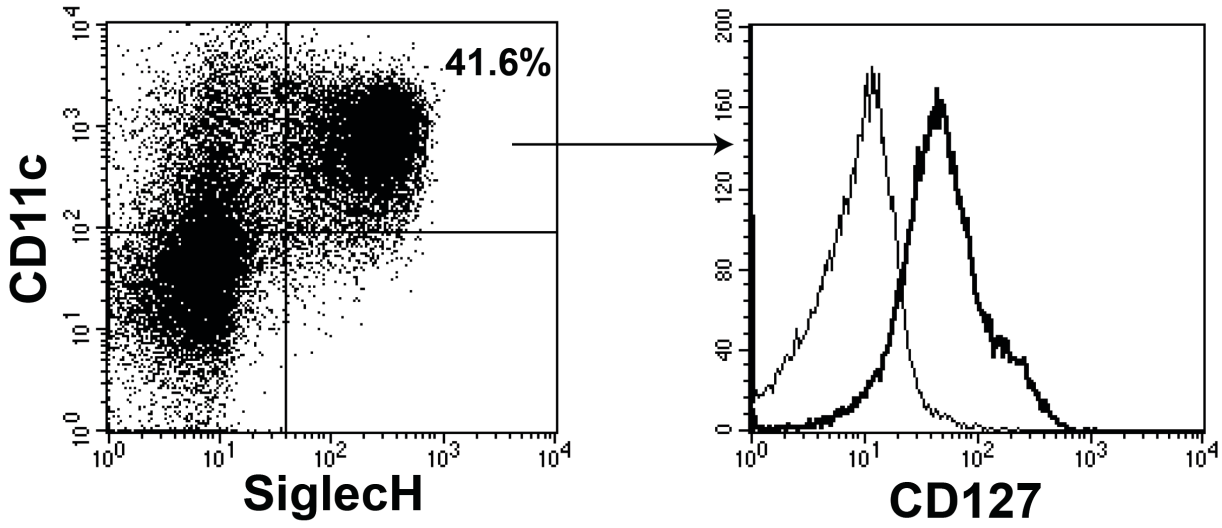
Levels of anti-NIP IgG as assessed by ELISA analysis in the blood of WT (white symbols) and FLT3L-Tg (black symbols) mice before (circles) and 13 days after (squares) immunization with NIP



Supplementary Figure 4

FLT3 expression in WT and FLT3-Tg hematopoietic progenitors.

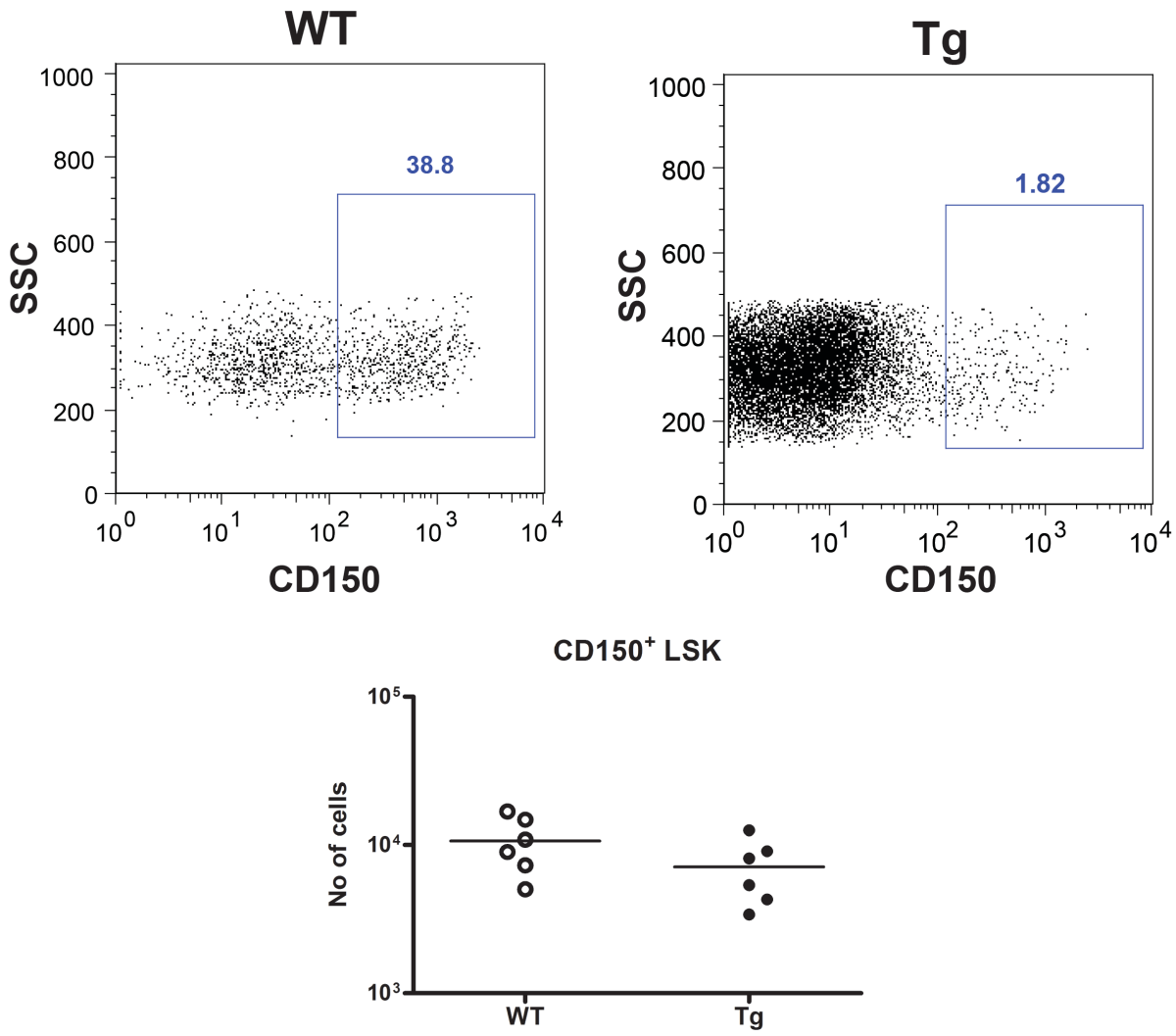
FACS stainings of hematopoietic progenitor populations in WT (red line) and FLT3L-Tg (blue line) mice. Cells were identified by FACS following the staining strategy showed in Figure 4 and additionally stained with an anti-FLT3 biotinylated antibody, followed by Streptavidin-PECy7 staining. Grey filled histogram: no anti-FLT3 antibody.



Supplementary Figure 5

CD127 expression on FLT3L-Tg pDC

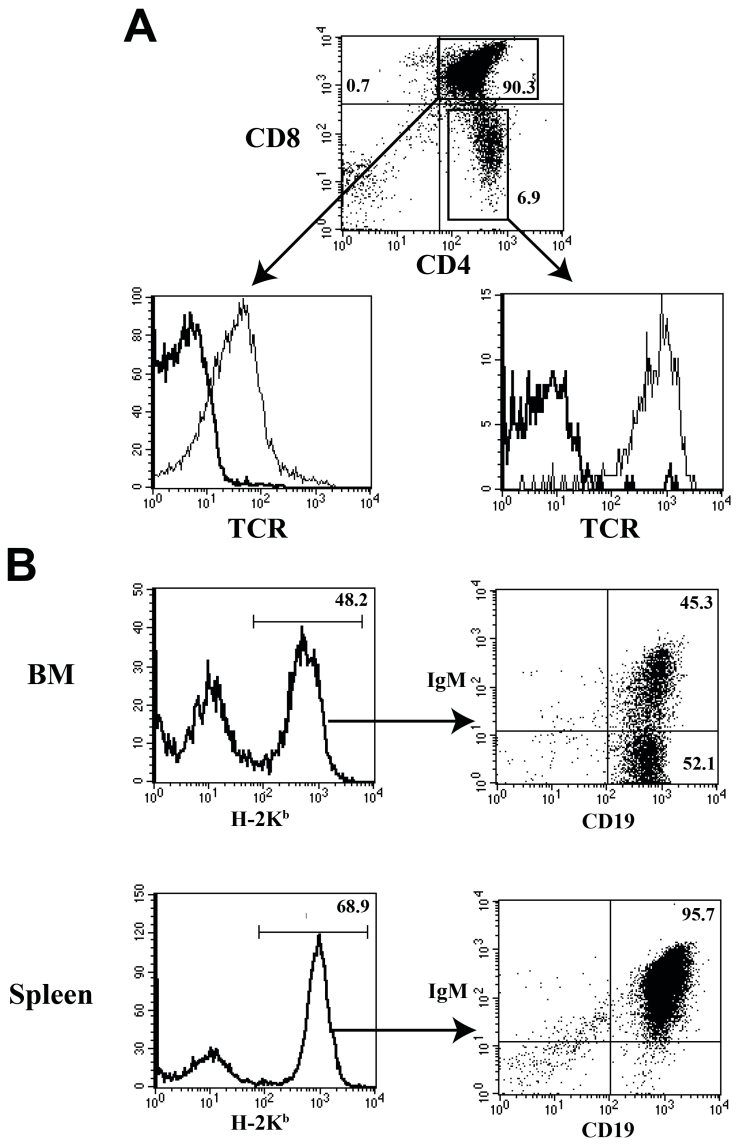
FACS analysis of $CD11c^{+}SiglecH^{+}$ pDC from FLT3L-Tg BM incubated with a biotinylated anti-CD127 antibody, followed by incubation with PE-labeled streptavidin. Thin line histogram: no anti-CD127 antibody, thick line histogram: staining with anti-CD127 antibody.



Supplementary Figure 6

CD150$^+$ LSK cells in WT and FLT3L-Tg mice.

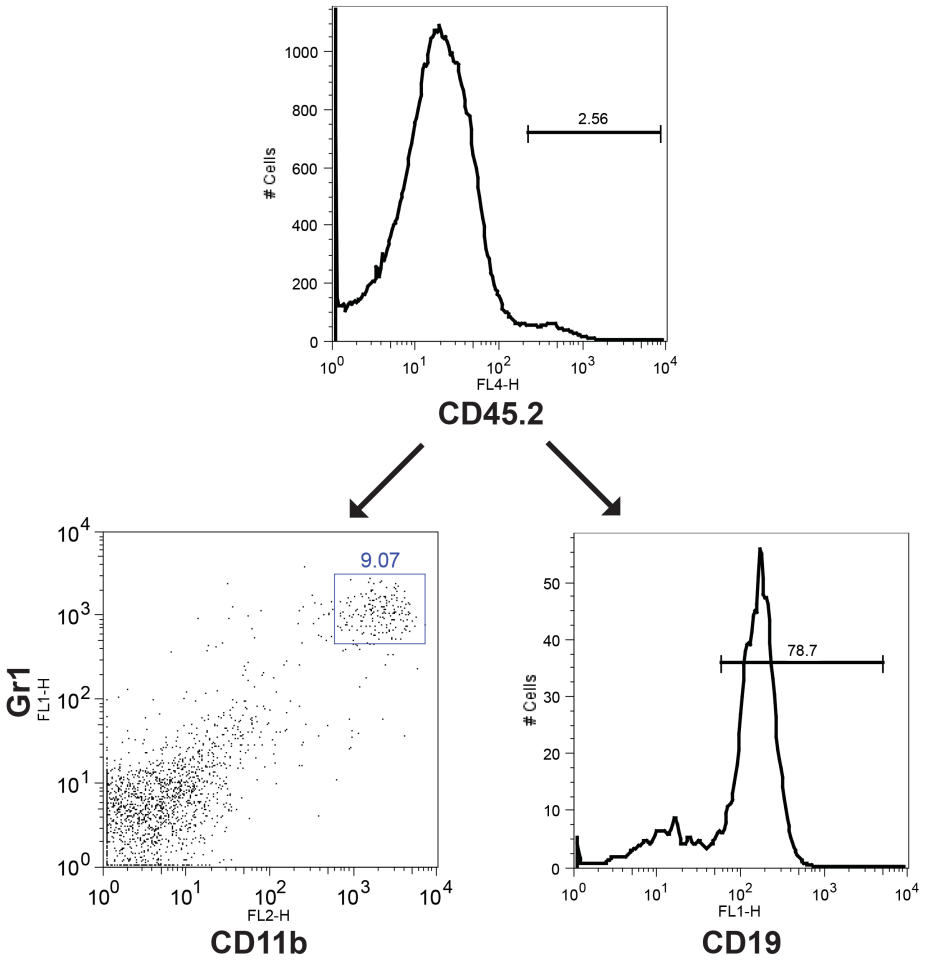
Upper panels: representative FACS plots of CD150$^+$ LSK cells in WT and FLT3L-Tg mice. LSK cells were stained as shown in Figure 4 and additionally stained with an anti-CD150-PE antibody. Percentages in gates indicate the percentage of CD150$^+$ cells within the corresponding LSK population. Lower panel: total numbers of CD150$^+$ LSK cells in WT (white circles) and FLT3L-Tg (black circles) mice (6 mice per group).



Supplementary Figure 7

B and T cell potential of FLT3L-Tg CLP.

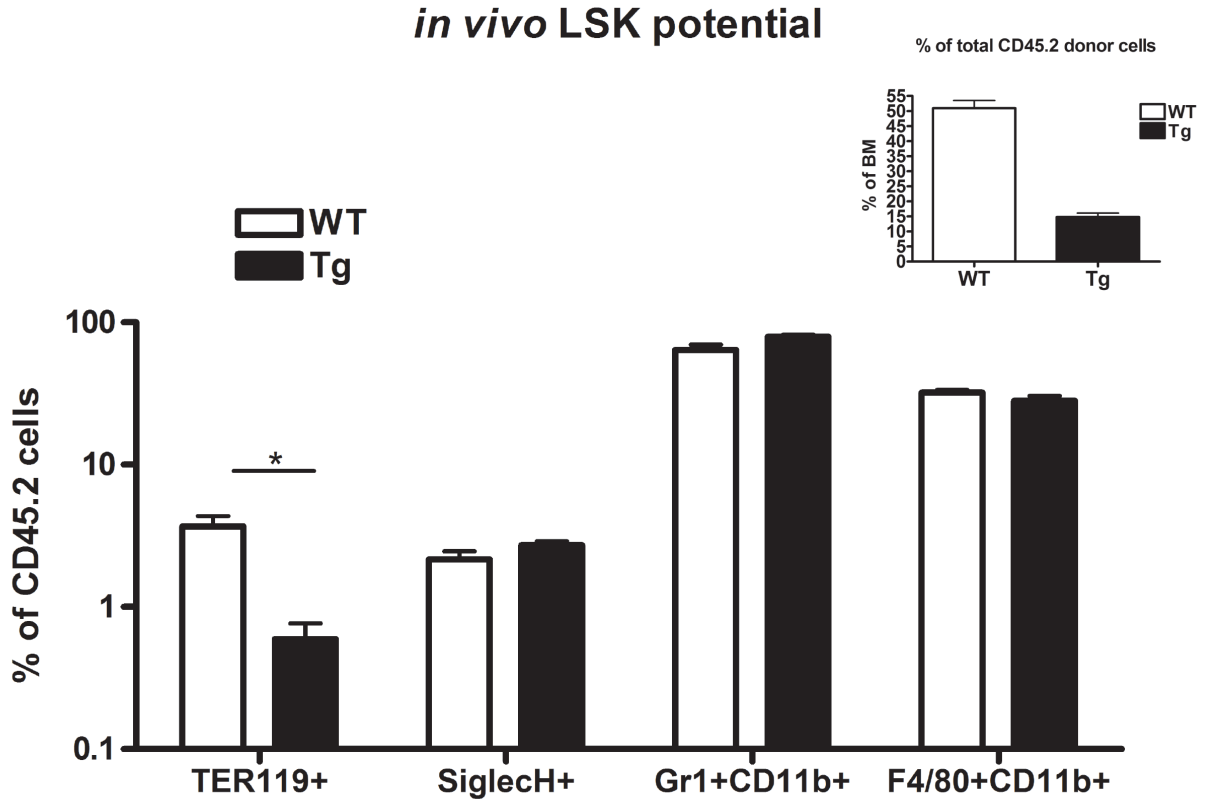
CD117$^+$Sca1$^+$CD127$^+$ CLP were sorted from FLT3L-Tg BM and intravenously transplanted into irradiated *Rag2*$^{-/-}$*γc*$^{-/-}$ recipients (50.000 CLP per mouse). Analysis of T and B cells was performed 3 weeks after transplantation. Figure shows analysis of one representative out of 4 recipient mice. A. CD4, CD8 and TCRβ staining of *Rag2*$^{-/-}$*γc*$^{-/-}$ thymus. B. CD19, IgM staining of *Rag2*$^{-/-}$*γc*$^{-/-}$ BM and spleen.



Supplementary Figure 8

B and myeloid *in vivo* potential of FLT3L-Tg EPLM

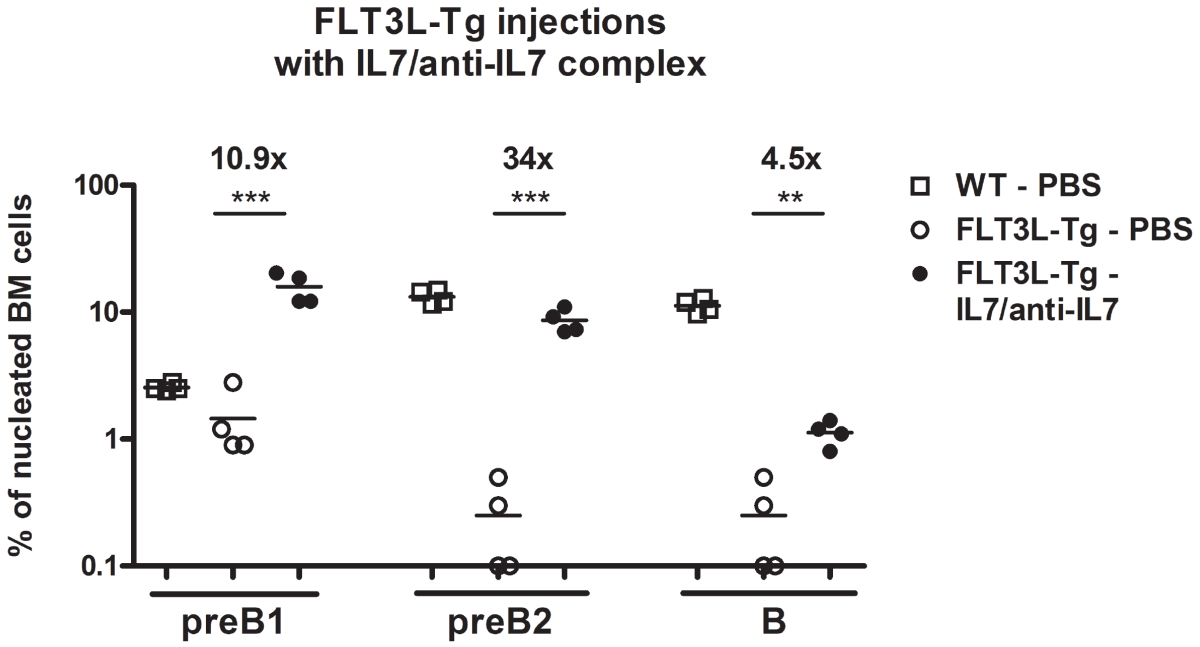
Thirty thousand EPLM were sorted from CD45.2 FLT3L-Tg BM and intravenously injected into congenic CD45.1 recipients together with 3 x 10^5 unfractionated CD45.1 BM cells, after lethal irradiation of the hosts. Spleens were analyzed 4 weeks following transplantation. Upper panel: percentage of CD45.2 donor cells in spleen. Lower panels: $CD45.2^+$ $CD19^+$ and $GR1^+CD11b^+$ cell stainings.



Supplementary Figure 9

Myeloid and erythroid *in vivo* potential of WT and FLT3L-Tg LSK cells.

Ten thousand WT and 15,000 FLT3L-Tg LSK cells (CD45.2) were sorted and intravenously injected into congenic CD45.1 recipients together with 3 x 105 unfractionated CD45.1 BM cells, after lethal irradiation of the hosts. BM were analyzed 7-10 days after transplantation and the percentage of the indicated hematopoietic cells within the CD45.2 compartment was calculated. For WT LSK 6 mice and for FLT3L-Tg 2 mice were transplanted. Upper-right panel: total percentage of $CD45.2^{+}$ cells at the time of the analysis. *:P=



Supplementary Figure 10

CD19$^+$ cell percentages following IL-7/anti-IL-7 complexes injections in mice

WT (squares) and FLT3L-Tg mice (circles) were injected with PBS (white symbols) or 15 µg/mouse IL-7/anti-IL-7 complexes (black symbols) three times with 3-day intervals. BM (2 *femurs* and 2 *tibias*) were analyzed 2 days after the last injection for the percentages of CD19$^+$CD117$^+$ (preB1), CD19$^+$CD117$^-$IgM$^-$ (preB2) and CD19$^+$IgM$^+$ (B) cells. ***(preB1): P=0.0006, ***(preB2): P=0.0001, **: P=0.0014.

Supplementary materials and methods

Generation of FLT3L-Tg mice.

Transgenic mice were initially generated in a C57BL/6 background. We have previously shown that in a model of C57BL/6 lymphocyte transplantation into (C57BL/6 x DBA/2)F1 hosts, treatment of the hosts with FLT3L provides protection against acute Graft Versus Host Disease(1). This prompted us to cross our C57BL/6 FLT3L-Tg mice to DBA/2 mice in order to obtain (C57BL/6 x DBA/2)F1 mice with sustained elevated levels of FLT3L. Subsequent analysis of both the C57BL/6 and (C57BL/6 x DBA/2)F1 genotypes revealed similar levels of FLT3L in their blood and an identical effect of FLT3L over-expression in hematopoietic populations (data not shown), with the exception of more profound splenomegaly in (C57BL/6 x DBA/2)F1 mice. All the data presented herein are from (C57BL/6 x DBA/2)F1 FLT3L-Tg mice, with the exception of certain transplantation experiments (Suppl. Figures 8 and 9) where CD45.2 C57BL/6 FLT3L-Tg mice were used.

Flow cytometry and cell sorting.

FITC-, PE-, APC- and biotin-conjugated monoclonal antibodies specific for CD11c, CD11b, CD117, CD19, CD127, CD115, CD8α, CD4, SiglecH, GR1, IgM, B220, Sca1, TER119, F4/80, CD34, CD16, FLT3, NK1.1, CD45.1 and CD45.2 were purchased from BD Biosciences or e-Biosciences or were made in our laboratory. Staining, flow cytometry and cell sorting were performed as previously described(2, 3).

In vitro limiting dilution assays.

OP9, OP9DL1 or ST2 stromal cells were plated in 96-well plates one day before the initiation of the experiment at 4000 cells per well. At the day of the experiment stromal cells were irradiated and subsequently co-cultured with hematopoietic progenitors at different concentrations. Cultures have been monitored with inverted microscope for generation of lymphoid or myeloid

cell colonies and after 2 (for OP9 cell cultures) or 3 weeks (for OP9DL1 and ST2 cell cultures) the total number of wells with no colonies was scored. For each experiment the number of wells with no colonies was plotted against the number of hematopoietic progenitors plated and the fraction of progenitor cells developing lymphoid or myeloid colonies was estimated.

Supplementary References

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II

Establishment of a stromal cell free culture system that allows the long-term propagation and proliferation of pro T cells, which can be used for the *in vivo* reconstitution of the T cell compartments

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Keywords:
T cell development, Notch ligand Delta-like 4 (DLL4), BM transplantation, Lymphopenia, Treg

Abbreviations

DLL1 Notch ligand Delta like 1
DLL4 Notch ligand Delta like 4
DN CD4 and CD8 double negative thymic T cell population
DP CD4 and CD8 double positive thymic T cell population
FL fetal liver
ISP immature single positive thymic T cell population
LSK lineage$^-$ Sca1$^+$ c-kit$^+$ fetal liver or adult BM haematopoietic progenitors
SP CD4 or CD8 single positive thymic T cell population

Summary

Lymphopenia following bone marrow transplantation or due to T cell loss during disease, e.g. HIV, impedes the efficient clearing of infections. Thus, ordinary infectious diseases pose a serious risk for patients with low T cell frequencies and novel approaches for therapy are needed.

We have developed an *in vitro* T cell culture system, which is not based on stromal cell lines and therefore is highly reproducable. To provide precursors with an adequate signal, a DLL4-Fc fusion protein is immobilized at the surface of cell culture flasks. Using this system, we studied the ability of surface-bound DLL4-Fc to induce prolonged and extensive expansion of *ex vivo* isolated $Sca1^+$ $c\text{-}kit^+$ fetal liver haematopoietic progentiors (LSK's) in presence of SCF and IL-7. *In vitro* generated pro T cells could be shown to reconstitute the thymi of irradiated T cell deficient *CD3ε*$^{-/-}$, T cell lymphopenic *preTalpha*$^{-/-}$ or WT mice and to generate a functional peripheral T cell compartment of both $CD8^+$ and $CD4^+$ antigen reactive T cells. However, reconstituted Rag- and CD3-deficient mice suffered from a wasting disease starting at week 5 to 8 following transplantation. Disease development could be prevented by co-injection of sorted $CD4^+$ $CD25^{high}$ wild type Treg cells. In a T cell sufficient (C57BL/6) or lymphopenic (*preTα*$^{-/-}$) setting, host Treg cells were sufficient to impede the development of disease. Thus, this *in vitro* culture system represents a highly reproducibel and powerful tool for the generation of large numbers of progenitor T cells suitable for transplantation and reconstitution of the T cell compartment maybe even in a clinical situation.

Introduction

Like all other cells of the hematopoietic system T cells are derived from hematopoietic stem cells (HSC's). However, unlike all the other cells of the hematopoietic system, T cell development takes place in the thymus and not in the bone marrow. The thymus does not harbor HSC's and therefore has to be constantly colonized by progenitor cells from the bone marrow in order to keep the generation of T cells intact [1-3]. Transplantation experiments have shown that various bone marrow progenitors can enter the thymus and can give rise to the generation of T cells [4], however the physiological role of these progenitors is still a matter of debate.

Based on the expression of the surface markers CD4 and CD8, T cell development in the thymus can be subdivided into 4 consecutive stages. The developmentally earliest ones are CD4 and CD8 negative and therefore called double negative (DN) cells. The DN's are the direct precursors of the CD4 and CD8 expressing cells called double positive (DP). Those that survive the positive and negative selection processes will then give rise to single positive CD4 or CD8 cells that are then allowed to leave the thymus and colonize the secondary lymphoid organs.

Based on the expression of CD25, CD44 and CD117, DN cells can be subdivided into 4 subpopulations. DN1 cells express high cell surface levels of CD44 and CD117, and are negative for CD25 whereas their DN2 progeny acquire the expression of all these 3 markers. DN3 cells express high levels of CD25 and low levels of both CD44 and CD117 [5-7]. It is at this stage of development that rearrangement of the TCRβ-chain locus is completed, and cells are selected for a productive TCRβ-chain rearrangement, also known as the β-selection checkpoint. The vast majority of DN3 cells that express a TCRβ polypeptide will undergo a preTCR-mediated proliferative expansion [8, 9] and will differentiate into DN4 cells. DN4 cells have lost the expression of CD25,

CD44 and CD117 and are the direct precursors of the immature single positive (ISP) CD8 cells [5-7]. ISP's will then differentiate into CD4- and CD8-expressing double positive (DP) cells. At this stage of T cell development, the TCRα–chain locus will be rearranged and those cells that are able to express a functional αβ TCR will undergo positive and negative selection [10, 11].

Several signaling pathways playing a crucial role in this complex T cell developmental program have been identified, and probably Notch signaling is the most crucial. Thus, it was shown that conditional inactivation of Notch 1 [12] or one of its ligands, Delta like ligand 4 (DLL4) [7, 13], resulted in a complete abrogation of T cell development in the thymus. Moreover, IL7-IL7R [14, 15] as well as stem cell factor (SCF)–c-kit [16, 17] signaling axis have been shown to be crucial for efficient T cell development. WNT and Sonic hedgehog have also been implicated in the development of T cells [18, 19] (and references herein) and additional signaling pathways might also be necessary for efficient T cell generation.

Several years ago Zuniga-Pflücker and colleagues generated an OP9 stromal cell line expressing the Notch ligand Delta like 1 (DLL1). This stromal cell line is able to promote differentiation into the T cell linage of early hematopoietic precursors [20, 21]. This culture system has been of great utility in our understanding of the early stages of thymocyte development. However, in such cultures, the density of DLL1 distribution and kinetics of Notch signal delivery are hard to control and additional signals, either cell surface or soluble delivered by OP9 cells remain unknown. Therefore, in order to identify the minimal requirements necessary for T cell commitment and differentiation we developed a stromal cell-free culture system. This culture system consists of plate bound DLL4, soluble SCF and IL-7. Lineage negative, Sca1 positive and c-kit positive cells (LSK's) differentiate under these

culture conditions into DN2 like cells. These cells can then be used for transplantation to reconstitute the T cell compartments of T cell deficient mice.

Results

Development of a stromal cell-free culture system for the study of T-cell differentiation.

Stromal cell-based culture systems have been useful for analyzing T cell development *in vitro* [20, 22]. However, several points emerged from using this culture system, the most notable being the heterogeneous response of cloned Pax-5 deficient pro B cells during their induction to differentiate towards the T cell lineage [23]. A possible reason for this heterogeneity was the variability in the strength and duration of Notch signaling. Therefore, in order to equalize the delivery of the Notch signals we prepared a soluble DLL4-Fc fusion protein [24-26]. However, initial experiments *in vitro* were not successful where soluble DLL4-Fc was added to the tissue culture medium containing progenitor cells. Therefore, we decided to fix the DLL4-Fc fusion protein to the surface of tissue culture plates via a mouse monoclonal antibody specific for the Fc-region of human IgG1. Thus, the mAb was coated to the plastic plates before addition of soluble DLL4-Fc (Fig. S1).

Using the OP9-DL1 stromal cell system, we previously showed that Pax-5 deficient pro B cells differentiating towards the T cell lineage up-regulates CD117 and down-regulates CD93 [17]. As shown in Fig. S2A these same cells undergo exactly the same phenotypic changes also on plate-bound DLL4. Optimal differentiation was achieved when plates were coated with 1μg/ml or more of DLL4-Fc (Fig. S2A).

The initial finding that non coated (soluble) DLL4-Fc did not induce the differentiation of Pax-5 deficient pro B cells towards the T cell lineage suggests that soluble DLL4-Fc even might inhibit T cell differentiation. In order to test this hypothesis, Pax-5 deficient pro B cells were cultured in plates coated with 2 μg/ml

DLL4-Fc in the presence of IL-7 (100U/ml) and SCF (20 ng/ml) together with various amounts of soluble DLL4-Fc.

As can be seen (Fig. S2B), differentiation could be completely inhibited at between 1 and 10μg/ml soluble DLL4-Fc added to the cultures. Thus, the blocking of Notch cross-linking efficiently inhibits the signalling induced by the plate-bound DLL4.

Now that differentiation of a cloned cell line towards the T cell lineage was homogeneous and occurred in a semi-synchronous fashion, it was decided to carry out gene expression analysis of cultured Pax 5 deficient pro B cells. In Figure S3A and B a summary of the T cell genes found to be up-regulated more then 3 fold and B cell genes found to be down-regulated more then 3 fold are shown. Thus, these findings strongly support the conclusion, that this stromal cell free culture system efficiently induces the development of Pax-5 deficient pro B cells towards the T cell lineage and moreover might well be an ideal system to identify Notch target genes necessary for T cell development.

Developing T cells from ex vivo isolated progenitors.

To further test this stromal cell-free culture system we initiated cultures with sorted undifferentiated hematopoietic progenitors, in this case lineage-negative, Sca-1^+, c-kit^+, (also called LSK) from fetal liver (FL) cells. Fifty thousand mouse LSK cells were sorted and cultured in 1 ml medium supplemented with 100U/ml IL-7 and 20 ng/ml SCF in 1 well of a 24 well plate coated with DLL4-Fc. After 5-6 days, cells became confluent and then re-plated in 2 times 4 ml IL-7 and SCF containing medium into 2 wells of a 6 well plate coated with DLL4-Fc. Thereafter, the cells were re-cultured at 2 x 10^5/ml into fresh DLL4-Fc coated plates every 4 days in medium containing the cytokines. Phenotypic analysis of the cells was performed at various

days after onset of the cultures. However, the phenotype of the growing cells did not change over time. An example of such a phenotypic analysis is shown in Figure 1A-C. Thus, a small fraction of cells express high levels of CD44 and low levels of CD25 (4.0% in this example). Yet, another small fraction expresses low levels of CD25 and no CD44 (1.6% in this example). All the other cells express high levels of CD25 and around 70% of those were also $CD44^{high}$, whereas the rest was CD44 negative. Moreover, all cells expressed CD117 and the level of this expression correlated with the CD44 expression (Fig. 1B). Cytoplasmic staining revealed that over 85% of the cells expressed CD3ε (Fig. 1C). No cytoplasmic TCRβ, nor cell surface CD4 or CD8 was detectable in or on these cells (data not shown). Next, we analysed Dβ1 – Jβ1 TCR rearrangements in these cells (Fig. 1D). As expected, all 7 rearrangements were readily detectable in total *ex vivo* isolated thymocytes and no germline band was detectable (lane 1). In marked contrast, Pax5 deficient pro B cells did not show any rearrangements but only the germline band (lane 2). In two independently established pro T cell lines (lanes 3 and 4) all 7 rearrangements were readily detectable but also the germline band was clearly visible. Based on these phenotypic and rearrangement analyses our *in vitro* propagated pro T cells seem to closely resemble DN2/3 thymocytes isolated *ex vivo*.

Moreover, we also analysed the long-term proliferative capacity of these pro T cells. A cumulative growth curve of a typical experiment of this nature is shown in Figure 1E. As can be seen, growth was exponential for the duration of the experiment, in this case 79 days, with cells expanding from 10^5 to 10^{24}, a 10^{19}-fold increase with an average doubling time of such cultures of about 1.5 days for the total culture. This finding indicates that these pro T cells have self-renewal capacity *in vitro*.

Additional non-TCRαβ subpopulations among in vitro-generated DN cells

NK and TCRγδ cells are frequently found among DN cells *in vivo* and the complete elimination of such cells from analysis and sorting is often difficult. To investgate this point further, we analysed LSK-derived *in vitro*-generated pro T cells further. As shown in Figure 2A, a distinct population of $NK1.1^+$ cells could be observed and these cells were brightly stained for CD44 (Fig. 2B) and were practically negative for CD25, i.e. are part of the DN1 population. After isolation by cell sorting, these cells killed the classical NK target YAC (Fig. 2E, dashed line) and their killing activity could be improved more than 10 fold after culture for 5 days in 100 U/ml of IL-2 (Fig. 2E, solid line). However, the sorted $NK1.1^+$ cells could not be propagated *in vitro* using plates coated with DLL4-Fc in the presence of IL-7 and SCF, suggesting that some DN1 cells constantly differentiate into NK cells but then die (data not shown). Likewise, TCRγδ-expressing cells were also present in these long-term cultures. Phenotypically, they were heterogeneous for CD44 expression and were to a large extent negative for CD25, i.e. are part of the DN1 and DN4 populations (Fig.2C and D). In order to test whether these TCRγδ-expressing cells could be propagated *in vitro* they were isolated by cell sorting and then cultured in DLL4-Fc coated plates or non-coated plates in the presence of IL-7 and SCF. As shown in Figure 2F, robust growth was observed in DLL4-Fc coated plates (solid line) but not in un-coated plates (dashed line). This finding suggests that part of TCRγδ-repetoire is Notch signaling dependent for its development and growth.

FL derived pro T cells differentiate into CD4/CD8 expressing cells upon withdrawal of IL-7.

Previously we showed that fetal derived pro T cells grown on OP9-DL1 stromal cells in the presence of IL-7 and SCF differentiated into CD4/CD8 expressing cells upon withdrawal of IL-7 [22]. More recently, Ikawa *et al* [26] reported a similar finding with fetal pro T cells grown in a stromal cell-free culture system. Now, we tested whether the pro T cells grown on DLL4 coated plates in the presence of IL-7 and SCF would also upregulate CD4 and CD8 expression upon IL-7 withdrawal. As shown in Figure 3A fetal liver derived pro T cells cultured for 5 days like this but in the absence of IL-7 differentiated into DP, and CD4 or CD8 single positive cells Additional staining with antibodies to TCRγδ or TCRβ indicated that gated DN cells (Fig. 3B) contained a few TCRγδ (dashed line) and TCRαβ (bold line) cells. DP cells (Fig. 3C) expressed TCRβ but not TCRγδ, whereas both single CD8 (Fig. 3D) and CD4 (Fig. 3E) subpopulations expressed TCRβ weakly with some TCRγδ cells present in the CD8SP population. Taken together, these results indicate that progression beyond the DN stage *in vitro* for fetal liver derived pro T cells was impeded by the presence of IL-7; a similar scenario is seen in pro B cell cultures *in vitro* where the presence of IL-7 prevents B cell differentiation into surface IgM expressing immature B cells [27].

In vivo T cell reconstitution of T cell deficient recipients by T cell progenitors generated in stromal cell-free cultures

Recently it was shown that pro T cells propagated on OP9-DL1 stromal cells were able to partly reconstitute the T cell compartments *in vivo* [28]. To test whether our stromal cell free generated pro T cells could reconstitute all T cell compartments *in vivo*, we performed the following experiment. CD45.1 CD3ε deficient mice were lightly irradiated (400 rad) and reconstituted intravenously with $5x10^6$ T cell

progenitors generated from fetal liver LSK's of CD45.2 mice cultured on plate bound DLL4-Fc in the presence of SCF and IL-7 for more than 5 weeks. As shown in Figure 4A (histogram), at 3 weeks after transfer, practically all thymocytes were CD45.2 postive. Moreover, these thymi contained all CD4 and CD8-defined subpopulations (Fig. 4B) and TCRβ was brightly expressed on the expected proportions of CD4- and CD8-defined subsets (Fig. 4C). In these experiments, reconstitution of the thymus was transient and the peripheral compartment (Fig. 4D) was still relatively lymphopenic (data not shown). Staining for FoxP3 in peripheral CD45.2 positive cells, revealed the complete absence of putative $CD4^+$ T regulatory cells (Fig. 4E).

As previously reported, after syngeneic BM transplantation into adult RAG- or CD3ε–deficient mice, T cell reconstitution of recipients thymi was readily demonstrable, but at between five and eight weeks, when mice were still relatively lymphopenic, mice died from a wasting disease marked by diarrhea, weight loss and signs of auto-immunity [29]. This phenotype may partly be explained by our findings of massive lymphocyte infiltrations in both liver (Fig 4F) and colon (Fig. 4G). We have also shown previously that this wasting/autoimmunity can be prevented by the co-injection of exogenous mature regulatory T cells (Treg) [29]. Therefore, to be able to test the functionality of the T cell compartment generated by the *in vitro* propagated pro-T cells, lightly irradiated (400 rad) adult CD45.1 CD3ε deficient mouse recipients were reconstituted with a mixture of $1x10^5$ *ex-vivo* sorted CD45.1 Treg cells ($CD4^+CD25^{high}$) from CD45.1 B6 mice and $5x10^6$ CD45.2 B6 T cell progenitors from *in vitro* cultures. As shown in Figure 5A, where peripheral blood was analysed, CD45.1 $CD4^+$ Treg cells (identified as CD45.2-negative, left population) could still be seen alongside CD45.2 donor progenitor-derived cells comprising both CD4 and CD8 lymphocytes (right population) at 10 weeks after

transfer. At this time after transfer, the mice remain healthy with no signs of wasting disease. Thus, provision of a small number of Treg cells protects adult mice from the development of autoimmunity following transplantation of progenitor T cells. In fact, upon analyses of the peripheral $CD4^+$ compartment, not only the injected Treg's ($CD45.1^+$) can be found, but now also have allowed the development of FoxP3 expressing cells among the injected 45.2^+ *in vitro* grown pro T cells (Fig. 5B).

In order to test whether these peripheral T cells are functional, mice were immunised with NIP-OVA in CFA. At day 14 after immunisation, the IgG anti-NIP antibody titer was determined. As shown in Figure 5C, the IgG anti-NIP titer in CD3ε-deficient mice injected with *in vitro* propagated pro T cells together with Treg cells was practically indistinguishable from that found in immunised normal B6 mice. In marked contrast, in CD3ε-deficient mice that had received only Treg, the anti-NIP antibody titer after immunisation was very low. Thus, the T cells derived from the *in vitro*-propagated pro T cells are functional in that they can help the antibody response to classical T-dependent antigen.

Long-term in vitro propagated pro T cells efficiently reconstitute T lymphopenic mice

PreTα deficient mice are highly T cell lymphopenic [8] and show an enrichment for $CD4^+CD25^{high}$ cells, many of which express FoxP3 and have a regulatory functions [30]. In order to test, whether our long-term *in vitro* grown pro T cells could fully reconstitute the peripheral T cell compartment of such mice, we transplanted $CD45.1^+$ *in vitro* grown pro T cells into sublethally irradiated $CD45.2^+$ *preTα*$^{-/-}$ mice. Analyses

of the spleen 6 weeks after transplantation show some 6% of the total lymphoid cells to be derived from the injected CD45.1$^+$ cells (Fig. 6A) showing the normal proportion of CD4 and CD8 single positive cells (Fig. 6B). The vast majority of these express the TCRβ-chain (Fig. 6C) and some 10-15% of the CD4$^+$ cells also express FoxP3 (Fig. 6D). Thus, in the presence of host derived CD4$^+$ Treg cells, our long term *in vitro* grown pro T cells show developmental capacities upon transplantation similar to normal *ex vivo* isolated progenitor cells.

Long term in vitro propagated pro T cells fully reconstitute all T cell compartments of lethally irradiated mice.

To investigate the developmental capacity of our *in vitro* grown pro T cells, we transplanted them to lethally irradiated CD45.1$^+$ B6 mice using an equal mixture of bone marrow from a CD45.1$^+$ CD3e$^{-/-}$ mouse and CD45.2$^+$ *in vitro* grown pro T cells. FACS analyses of thymi and spleens was performed 3 weeks after reconstitution and is shown in Fig. 7. Virtually all thymocytes are CD45.2$^+$ (Fig. 7A) with the expected CD4- and CD8-defined subpopulations (Fig. 7B) with TCRβ brightly expressed on the expected proportions of SP thymocytes (Fig. 7C). The spleens showed some 15-20% donor derived cells (Fig. 7D) with the majority (more than 80%) being CD4 and CD8 single positive cells (Fig. 7E) expressing the TCRβ at the surface (Fig. 7F). Some 10-15% of the donor derived CD4$^+$ cells also express FoxP3 (Fig. 7G). The spleens contain the normal proportions of donor derived γδ T cells as well as NK1.1 positive cells (Fig. 7H). Thus, our *in vitro* propagated pro T cells efficiently reconstitute all T cell compartments of lethally irradisted wild type mice.

Discussion

Here we report on a novel tissue culture system not requiring stromal cells for the long-term propagation of mouse progenitor T cells. The system employes tissue culture dishes with surface-bound Delta Like Ligand 4 (DLL4) for propagation of progenitor cells in the presence of SCF and IL-7. The extracellular domain of mouse DLL4 is produced as a soluble fusion protein containing the Fc fragment of human IgG_1 so as to allow binding in the right orientation to tissue culture plastic plates previosly coated with a mouse anti-human Fc specific monoclonal antibody. Following the initiation of cultures with sorted fetal liver derived multi-potent LSK progenitors incubated with IL-7 and SCF in such DLL4-coated plates, a continuous expansion of cells was seen for up to six months and beyond, providing that cells are replated at 10^5 cells/ml every 4 days onto freshly DLL4-coated plates in medium containg SCF and IL-7. During this time, the number of cells doubeled every 30 hours and remained at a constant ratio of a mixture of DN1-like and DN4-like cells. Thus, given that the minimum cell cycle time is 30 hours or less, such cultured cells have passed well beyond the so called Heyflick limit [31] without going through any noticeble "crises" (Fig. 1E) and remain stricly dependent on simultanous signalling by DLL4 and the cytokines SCF and IL-7.

For the most part, differentiation was uni-directional, however, sorting experiments indicated that $CD44^+CD25^+$ (DN2) cells generated *in vitro* could relatively rapidly re-adopt a $CD44^+CD25^-$ DN1 phenotype (data not shown). During normal T lineage differentiation in the thymus, intracytoplasmic CD3ε protein begins to be expressed among DN2 cells with complete expression being achieved at the DN3 stage. Following V to DJβ gene rearrangements at the DN3 stage, intracytoplasmic TCRβ protein appears in DN3 cells and then, following the process of so-called TCRβ

selection, all post-DN3 TCRαβ lineage cells are universally intracytoplasmic TCRβ^{+}.

Analysis of intracytoplasmic CD3ε and TCRβ expression among *in vitro*-grown DN cells indicated some differences with this *in vivo* pattern. Notably, DN1 cells in this *in vitro* system contained a disctinct population of intracytoplasmic CD3ε^{+} cells, again presumably indicative of some degree of de-differentiation from intracytoplasmic CD3ε^{+} DN2 cells. In addition, unlike their *in vivo* counterparts, DN4 cells contained many i.c. TCRβ^{-}, probably TCRγδ cells.

Additional subpopulations of TCRγδ and NK cells could be found among cultured DN cells. For NK cells, these mostly appeared to have a DN1-like phenotype whereas TCRγδ cells were a mixture of mostly DN1 and DN4 cells. The presence of of a significant proportion of TCRγδ cells could account for both the intracytoplasmic CD3ε^{+} DN1 and TCRβ^{-} DN4 cells. Previously it had been shown that some human NK clones are intracytoplasmic CD3ε^{+} [32]. It could well be that mouse DN1-like NK cells show the same and thus could also account for some intracytoplasmic CD3ε^{+} DN1 cells. In the presence of IL-7, cells remained DN, but upon withrawal of IL-7, fetal liver derived pro T cells acquired both CD4 and CD8 surface antigens, differentiating to DP cells and later to CD4^{+} or CD8^{low} SP cells. The latter were clearly not mature cells in that they expressed very low levels of CD3ε and TCRβ and did not respond to anti-CD3 stimulation (data not shown). Thus, like in cultures containing Notch-ligand expressing stromal cells [22] differentiation of T cells is incomplete also in the culture system described herein.

Bone marrow transplantation is a frequently used treatment modality in medical practice. However, following infusion of HSC, reconstitution of the T lymphocyte compartment is often delayed and protracted because of the lag in thymus colonisation by donor-derived precursors [33]. In mouse model systems, we have encountered additional difficulties of T cell reonstitution with *in vitro*-generated T cell progenitors, difficulties related to Treg. In essence, during the initial stages of reconstitution of T cell deficient recipient mice using either T cell-depleted bone marrow preparations or purified T cell progenitors, although the thymus is adequately re-colonized, the peripheral T cell compartment contain low numbers of Treg cells. After about four weeks, the imbalance between Treg numbers and newly produced naïve T cells results in the development of auto-immune disease marked by weight loss, wasting, diarrhea and death of mice [29]. We have shown that co-injection of *ex vivo* isolated $CD4^+$ $CD25^{high}$ wild type Treg cells can rescue T cell deficient recipient mice from autoimmunity and death. In this study, we have carried out some additional experiments to explore the Treg issue. Confirming our previous results, reconstitution of irradiated lymphopenic mice with *in vitro*-generated T cell progenitors results in robust thymus reconstitution with phenotypically mature T cells. This result indicates firstly that T cell progenitors generated in a stromal cell-free culture system retain the ability to home to the recipient thymus. Secondly, it shows that differentiation of *in vitro*-generated progenitors proceeds to completion in the thymus *in vivo*, with the development of fully functional $TCR\alpha\beta^{high}$ single positive cells. Even though TCR^{high} $CD4^+$ cells can be found in the lymphoid organs and blood of adult T cell deficient recipients (Fig. 4), the proportion of Treg is insufficient to protect mice from auto-immunity. Interestingly, use of lymphopenic recipients overcomes this problem with approximately 20% TCR^{high}, CD4 PBL T cells being Treg (Fig. 5).

Nevertheless, our experiments clearly show that to rescue adult T cell deficient recipients, co-injection of small numbers of additional Tregs is necessary to avoid the development of auto-immunity (Fig. 5).

In conclusion, we have shown that practically limitless numbers of functional T cell progenitors can be generated from multi-potent fetal liver derived progenitors in an *in vitro* system containing the cytokines IL-7 and SCF without the need for stromal cells. Such cells can be frozen and thawed and as previously shown for stromal cell-generated progenitors, can be genetically manipulated for therapeutic purposes. Using this system for human progenitors might offer numerous therapeutic applications.

Material and Methods

Mice

Female C57BL/6 45.1, C57BL/6 45.2 and CD3ε deficient [34] as well as preTα deficient [8] mice of 5–8 weeks of age were used. All mice were bred and maintained in our animal facility under specific pathogen-free conditions. All animal experiments were carried out within institutional guidelines (authorization number 1888 from Kantonales Veterinäramt, Basel).

Cell lines

$Pax5^{-/-}$ fetal liver derived pro B cells were generated and maintained on OP9 stromal cells in the presence of IL-7 as previously described [35].

Recombinant DLL4-Fc fusion protein

The production and purification of a fusion protein consisting of the extracellular portion of mouse DLL4 (amino acids 1–521) and the Fc portion of human IgG1 has been described before [36].

Monoclonal antibody against human IgG1 Fc

A mouse monoclonal IgG antibody against the Fc part of human IgG_1 (clone Huf5.4) was produced by conventional techniques using the fusion partner Sp2/0.

Flow cytometry and cell sorting

FITC-, PE-, APC- or biotin-labeled mAb specific for CD3, CD4, CD8α, CD25, CD45.1, CD45.2, CD117, Sca-1, TCRβ, TCRγδ and NK1.1 were purchased from BD Biosciences or e-Biosciences. Staining of the cells was performed as described [23]. Flow cytometry was performed using a FACS Calibur (BD Biosciences) and data were analyzed using the Cell Quest Pro Software (BD Biosciences). For cell sorting, the FACS Aria (BD Biosciences) was used. Re-analysis of the sorted cells indicated

that in all instances they were over 98 % pure. LSK's were defined and sorted as previously described [37-39].

Cell cultures

In initial experiments, 48- or 24-well tissue culture plates from Nunc (Nunc A/S, Roskilde, Denmark) were used. Once established, cells were maintained in 6-well bacterial grade plates (Greiner Bio-One, Kremsmünster, Austria), because of their higher coating capacity. Wells were pre-coated overnight or longer with 10 μg/ml mAb anti-human IgG_1-Fc in PBS (0.25 mL per well for 48 well plates, 0.5ml per well for 24 well plates or 2 ml per well for 6 well plates) at 4°C. Thereafter, wells were washed twice with IMDM (BD Biosciences) supplemented with 5 - 10 % FCS, $5x10^{-5}$M β-mercaptoethanol, 1mM glutamine, 0.03 % w/v Primatone (Quest, Naarden, The Netherlands), 100 U/ml penicillin, 2 % serum (complete medium) and then used for coating with DLL4-Fc at 2 μg/ml (or as indicated) in IMDM overnight, washed and used for cell culture.

NK cell cytotoxicity assay

NK cell cytotoxic activity was determined using YAC-1 cells as targets [40]. The assay was performed as previously described [23].

PCR analyses of TCRβ gene rearrangements

DNA was isolated from indicated cells and analysed by PCR for Dβ1 to Jβ1 rearrangements as described before [39].

Gene expression pattern of a cultured pro B cells

Isolation and processing of RNA from Pax 5-/- pro B cells cultured as described in Fig. S3 as well as its analyses on Affymetrix Mouse Genome 1.0 ST gene array was performed as previously described [41].

Transfers of cultured progenitor cells

Recipient mice were irradiated as indicated using a ^{137}Cs source. Sorted populations were infused intravenously. Spleen and marrow from one femur were collected separately from recipient mice, and donor- and host-derived cells were assayed by flow cytometry.

4-Hydroxy-5-iodo-3-nitrophenyl (NIP)-specific IgG responses

Mice were immunized subcutaneously at day 0 with 100 μg NIP-OVA in a 1:1 complete Freunds adjuvant (CFA) emulsion and serum IgG against NIP was analyzed at day 14 using ELISA as described in [42].

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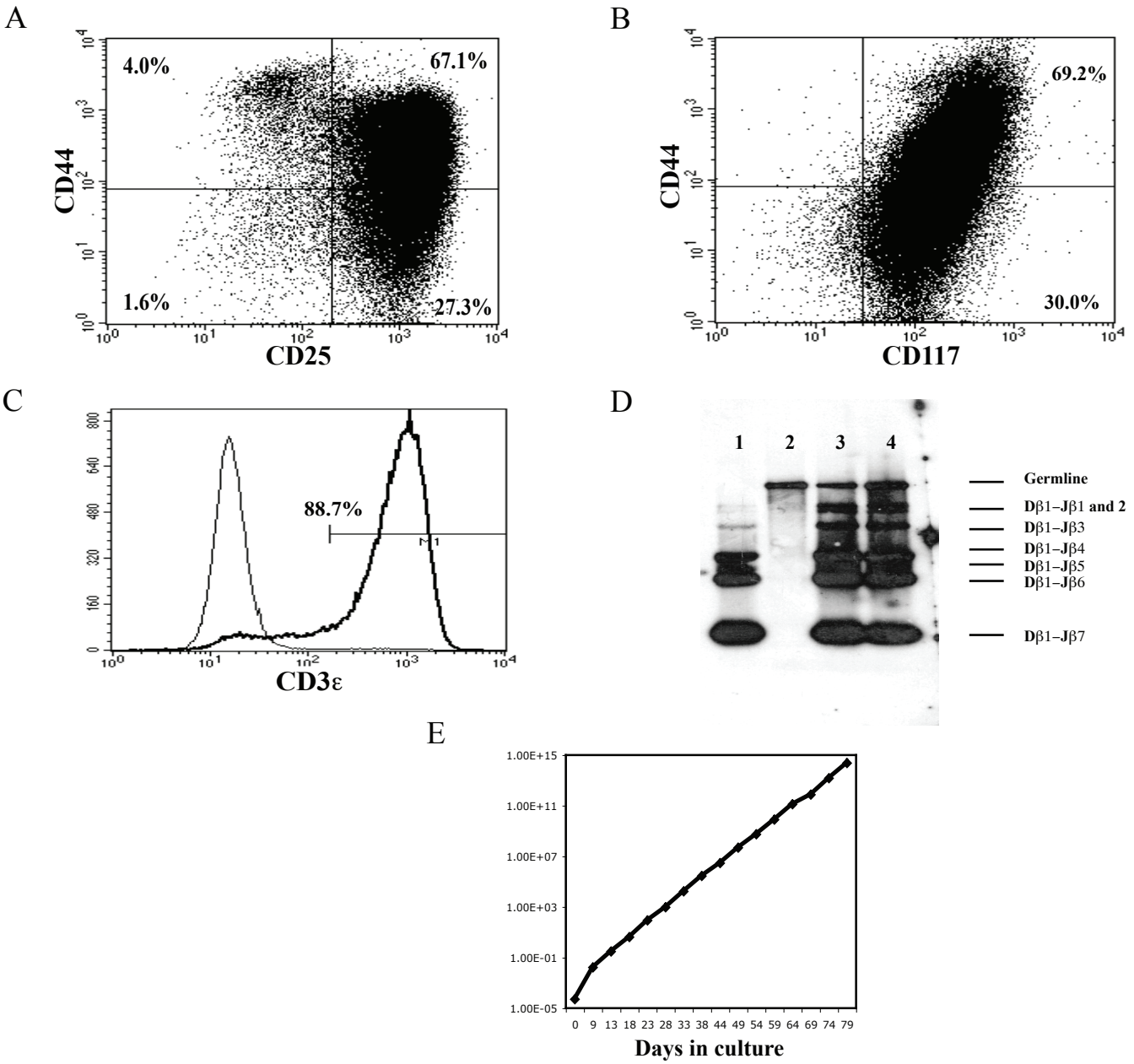

Figure 1. *Phenotype, genotype and proliferative capacity of LSK derived pro T cells.*
Fetal liver derived LSK's were cultured for 55 days on plate-bound DLL4 in the presence of SCF and IL-7. (A) Double staining for CD44 and CD25. (B) Double staining for CD44 and CD117. (C) Intracellular staining for CD3ε in CD25 gated cells. (D) PCR analyses of Dβ1 to Jβ1 rearrangements in genomic DNA isolated from wild type thymus (lane 1), Pax5$^{-/-}$ pro B cells (lane 2) and two samples of in vitro generated pro T cells (lanes 3 and 4). (E) Cumulative growth curve of pro T cells propagated on plate-bound DLL4.

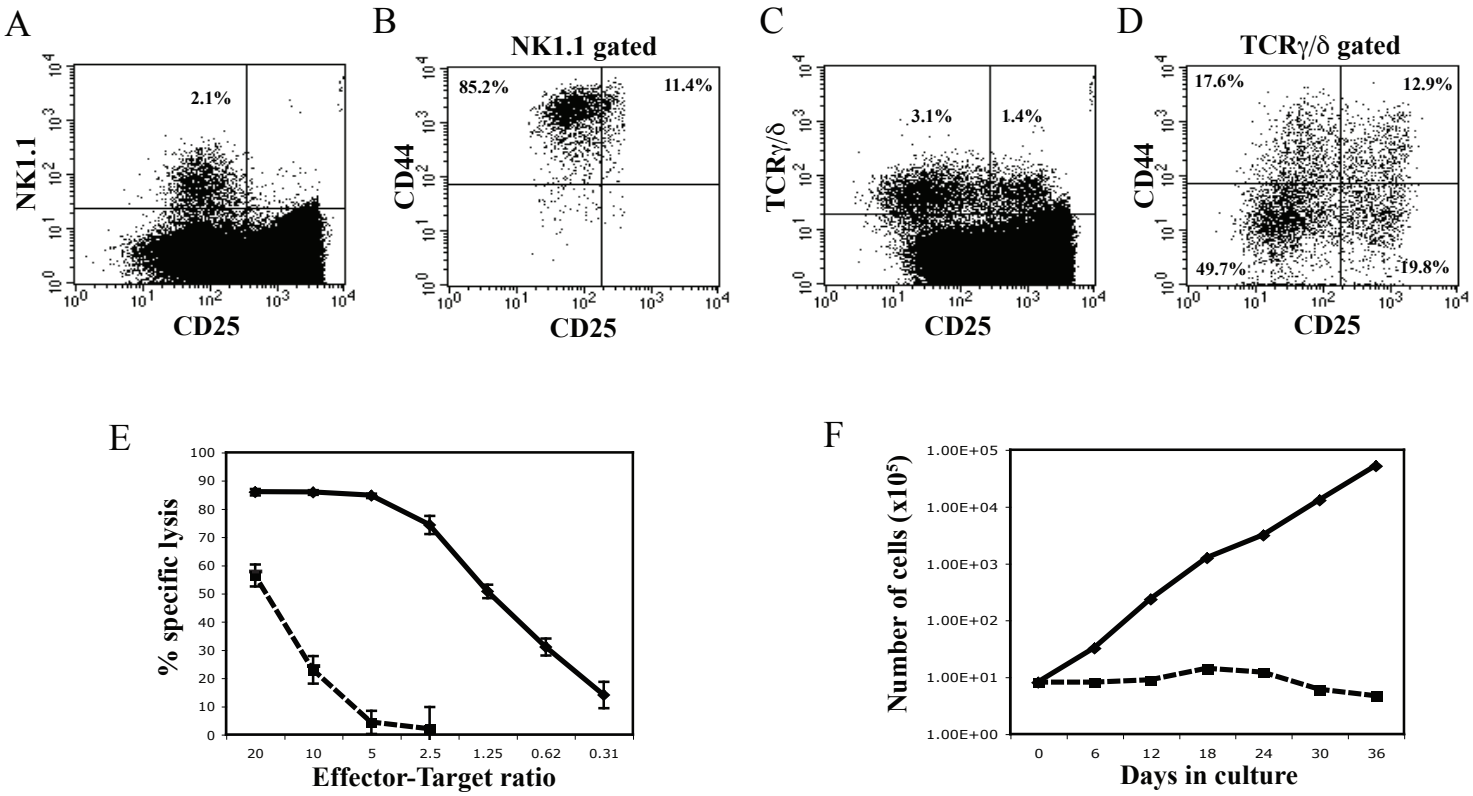

Figure 2. *Co-development of NK cells and γδ T cells during culture of LSK's on plate-bound DLL4*.
Long-term cultured pro T cells were stained for CD44 and NK1.1 (A) as well as CD25 (B). A second staining for CD44 and TCRγδ (C) as well as CD25 (D) was performed on cells from the same culture. Sorted NK1.1 positive cells were assayed for cytotoxic activity against YAC-1 target cells (E) either directly after sorting (dashed line) or after 5 days of culture in the presence of IL-2 (solid line). Cumulative numbers of sorted TCR γδ positive cells were cultured (F) in the presence of IL-7 and SCF on plates coated (solid line) or not coated (dashed line) with DLL4.

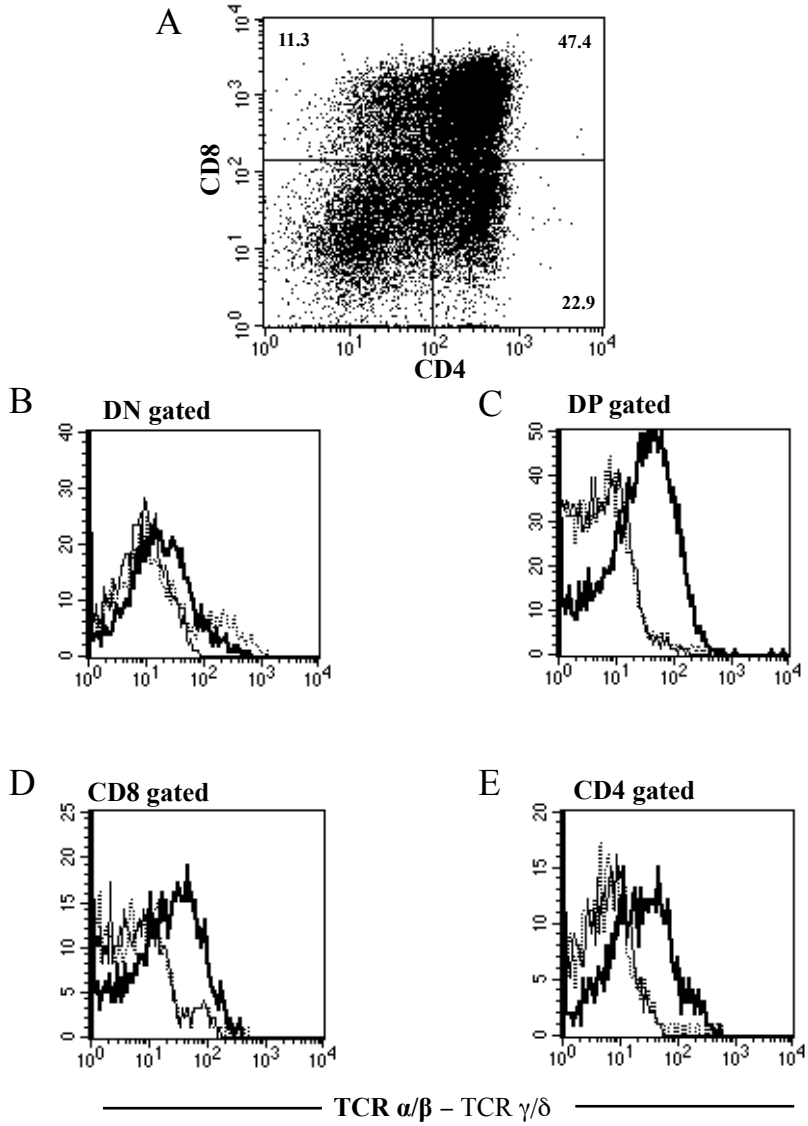

Figure 3. *Fetal liver derived pro T cells differentiate into CD4 and CD8 expressing cells upon withdrawal of IL-7.*

Long term grown pro T cells were washed and cultured in DLL4 coated plates in the presence of SCF but without IL-7 for 5 days. Cells were analysed for surface expression of CD4 and CD8 (A) as well as TCRαβ and TCRγδ (B-E). Bold lines in the histograms show staining for TCRβ, dashed line for TCRγδ and thin lines indicate auto-fluorescence of unstained cells.

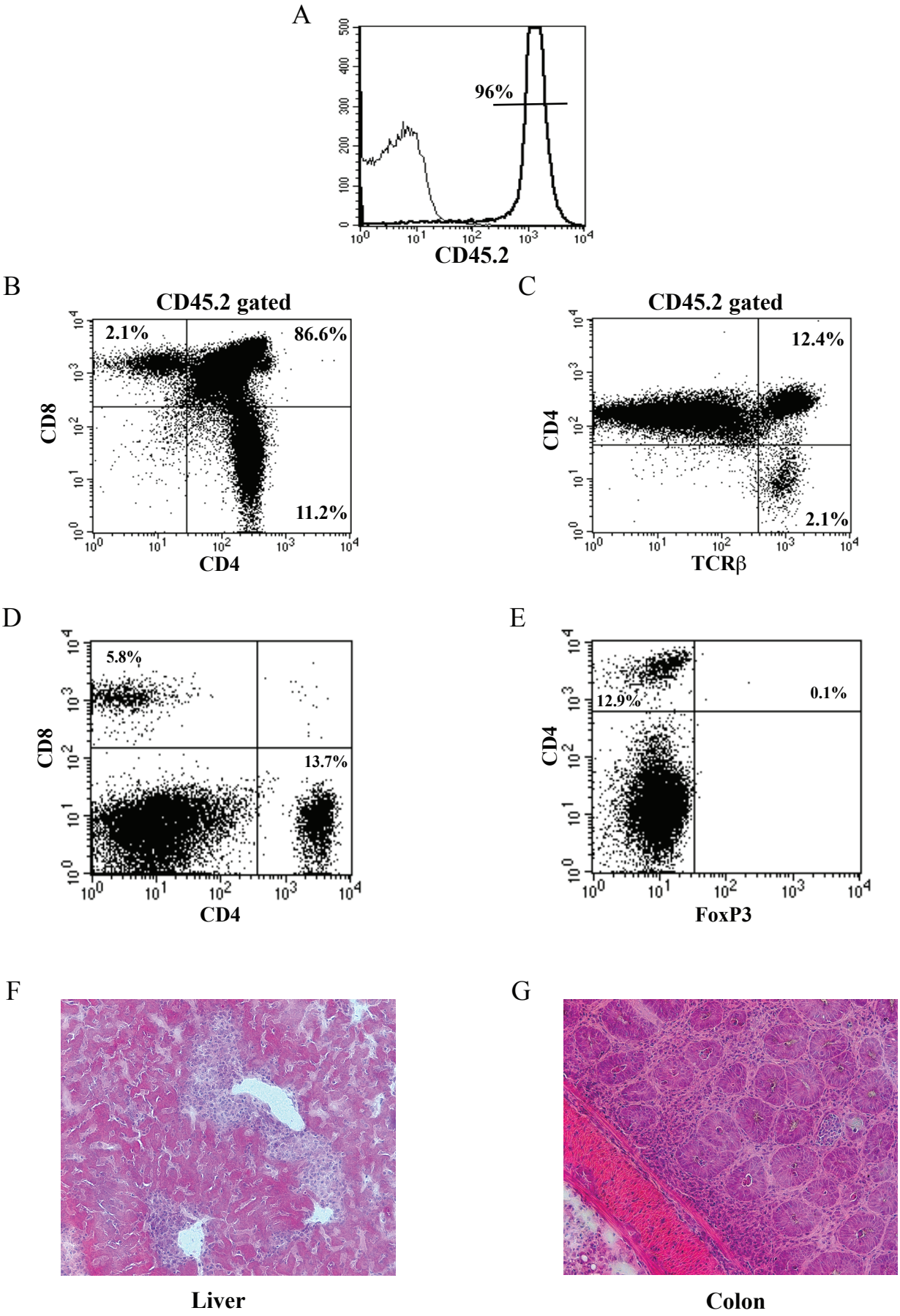

Figure 4. *In vivo T cell reconstitution of T cell deficient recipients by T cell progenitors generated in stromal cell-free cultures*.

CD45.1$^+$ CD3ε deficient mice were sublethally (400R) irradiated and reconstituted with 5x10^6 long-term *in vitro* propagated pro T cells. Three weeks after reconstitution, cells of the thymus (A-C) and spleen (D, E) were analysed by FACS for donor derived CD45.2$^+$ cells. (A) Thymocytes were gated on CD45.2$^+$ cells and analyzed for the expression of CD4, CD8 (B) and TCRβ (C). Spleen cells from the same animal were analysed for CD4, CD8 (D) and FoxP3 (E). Frozen sections of liver (F) and colon (G) from the same animal were stained with H/E and shown at 50X magnification.

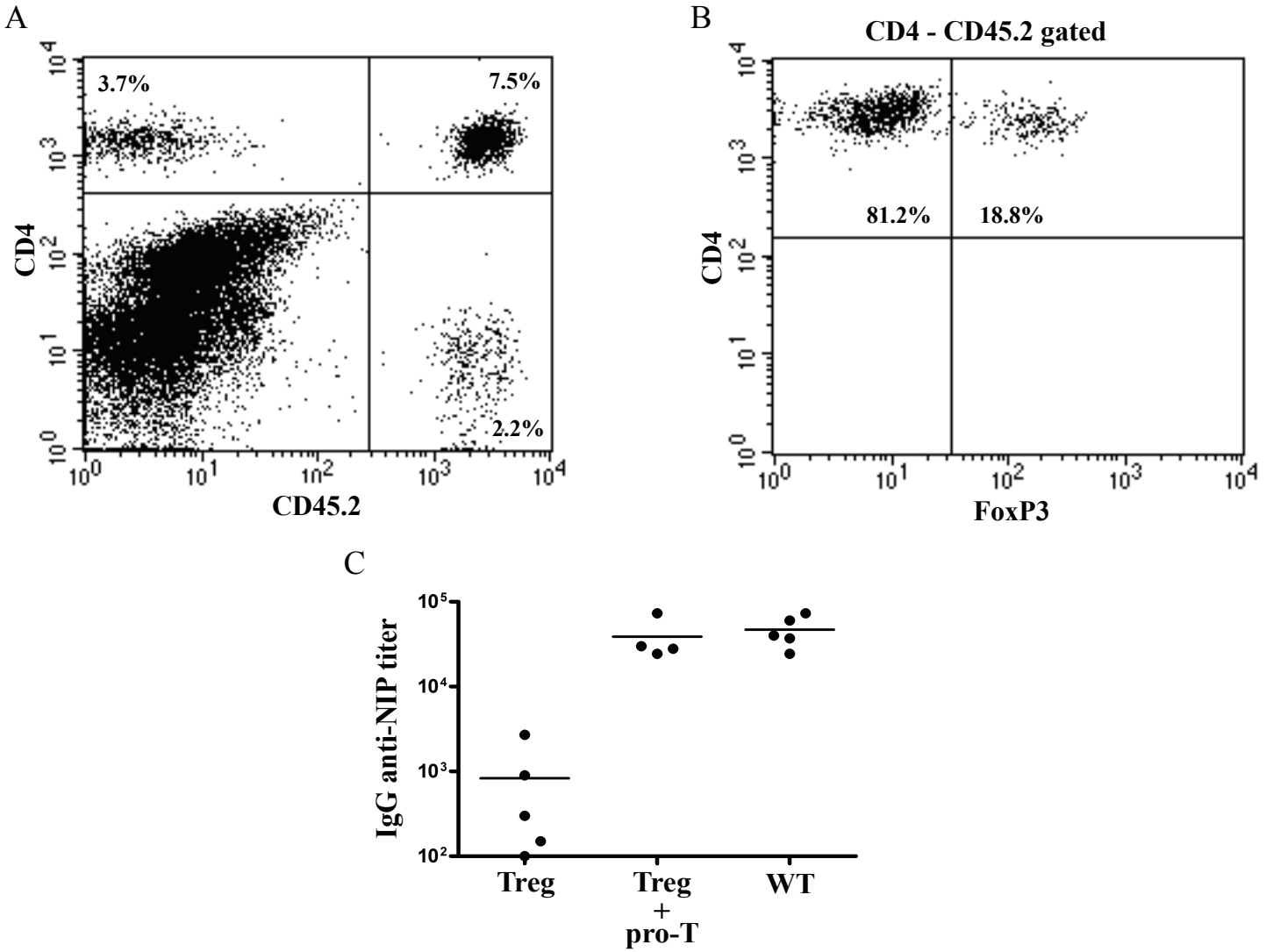

Figure 5. *Prevention of autoimmunity and wasting in T cell deficient recipients by co-injection of Treg's together with T cell progenitors*.
CD45.1$^+$ CD3ε deficient mice were sublethally (400 rad) irradiated and reconstituted with a mixture of 10^5 CD45.1$^+$ CD4$^+$ CD25$^+$ cells and $5x10^6$ CD45.2$^+$ fetal liver derived, long-term in vitro propagated pro T cells. 10 weeks after reconstitution, peripheral blood was analysed for CD4$^+$ cells (A) and CD45.2$^+$ FoxP3 expressing cells (B). At the time for blood analyses, reconstituted CD3ε deficient mice and wild type C57BL/6 mice were immunized with NIP-OVA and their IgG anti-NIPA serum titers determined 14 days later (C).

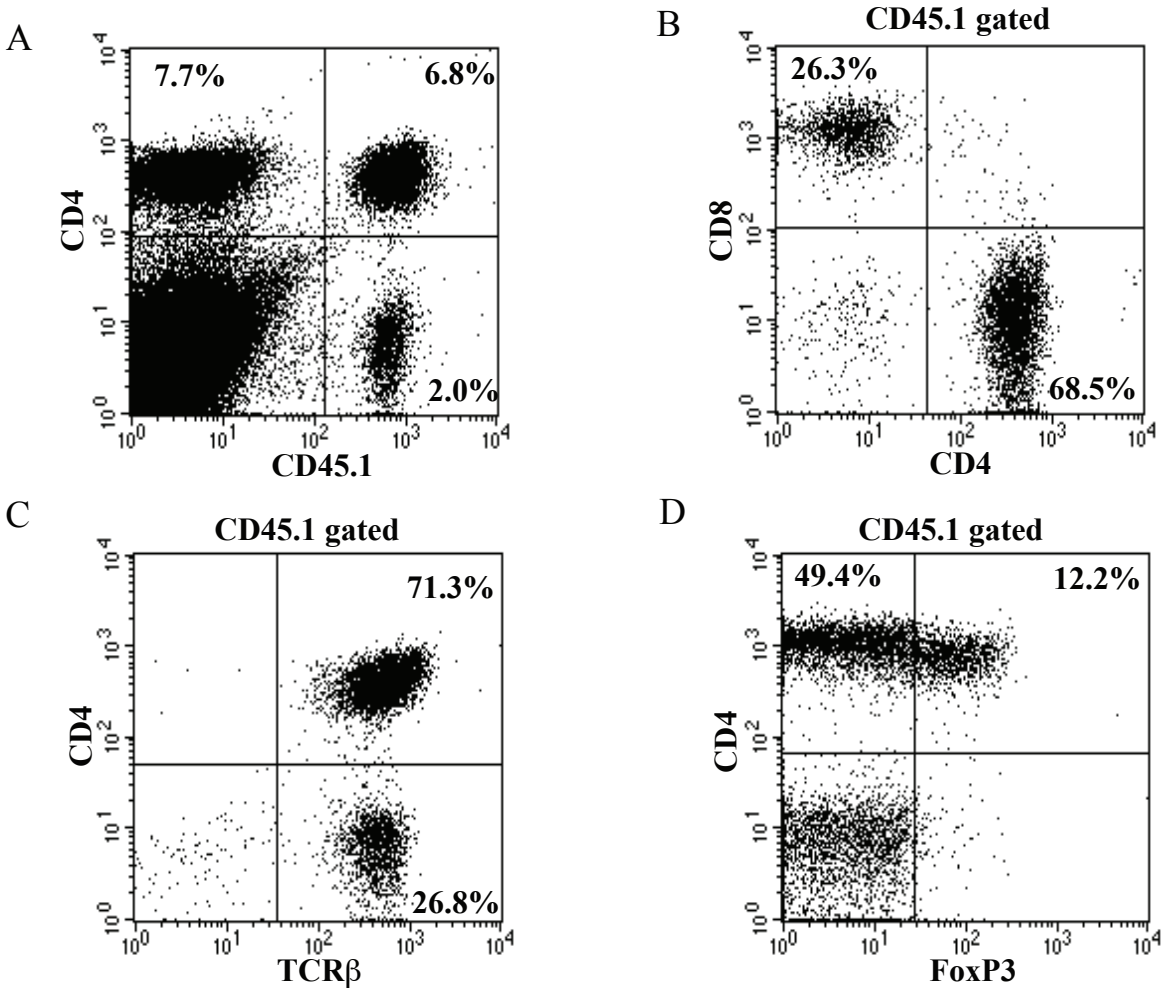

Figure 6. *In vitro generated pro T cells can reconstitute the effector and regulatory T cell repertoire in lymphopenic mice*.

(A) Lymphopenic CD45.2^{+} preTα deficient mice were sublethally irradiated (400 rad) and transplanted with CD45.1^{+} in vitro generated pro T cells. Reconstitution of the T cell repertoire in the spleen by host and pro T cells could be shown 6 weeks after transplantation. Analysis of the pro T cell (CD45.1^{+}) derived T cell compartment revealed CD4 and CD8 (B), CD4 and TCRβ (C) and CD4 and FoxP3 (D) expression.

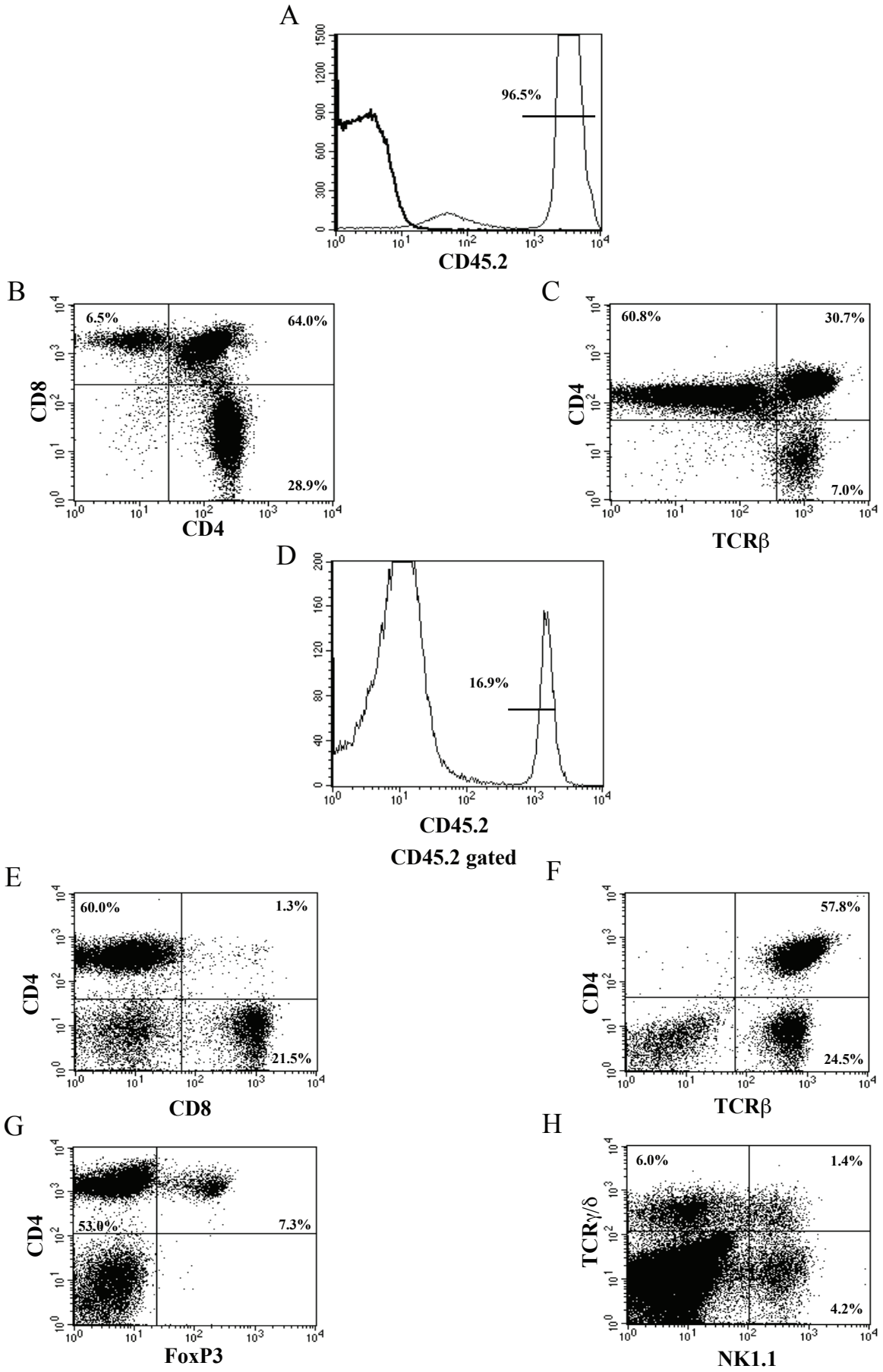

Figure 7. *In vitro generated pro T cells can reconstitute the T cell repertoire in a bone marrow transplantation model*.

CD 45.1$^+$ C57BL/6 wild type mice were lethally irradiated (950 rad) and reconstituted with an equal mixture of bone marrow from CD45.1$^+$ CD3$\varepsilon^{-/-}$ mice and CD45.2$^+$ in vitro generated pro T cells. 3 weeks after transplantation thymus reconstitution by CD45.2$^+$ in vitro generated pro T cells was analyzed (A). Expression of CD4 and CD8 (B) or CD4 and TCRβ (C) on CD45.2$^+$ thymocytes is shown in the respective dot plot. Reconstitution of the peripheral T cell compartment in the spleen by CD45.2$^+$ in vitro generated pro T cells was analyzed at week 3 (D). Expression of CD4 and CD8 (E), CD4 and TCRβ (F), CD4 and FoxP3 (G) and TCRγδ versus NK1.1 (H) on CD45.2$^+$ splenocytes is shown.

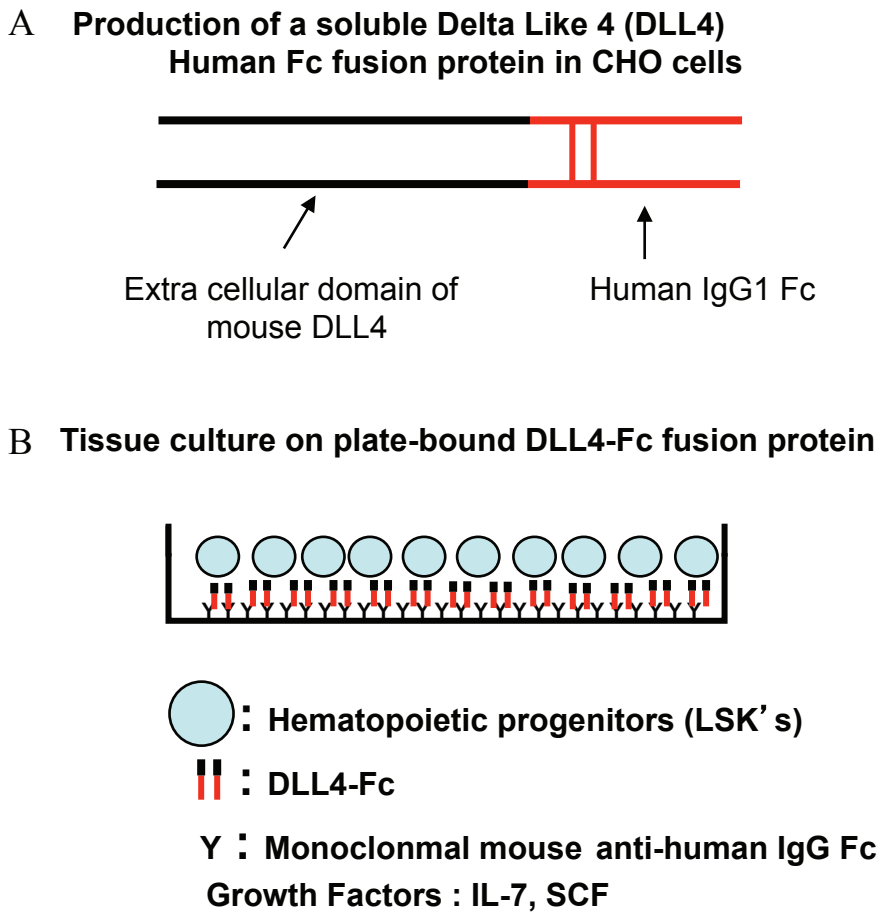

Fig. S1. *Establishment of culture conditions, that allows the long-term propagation of pro T cells.*

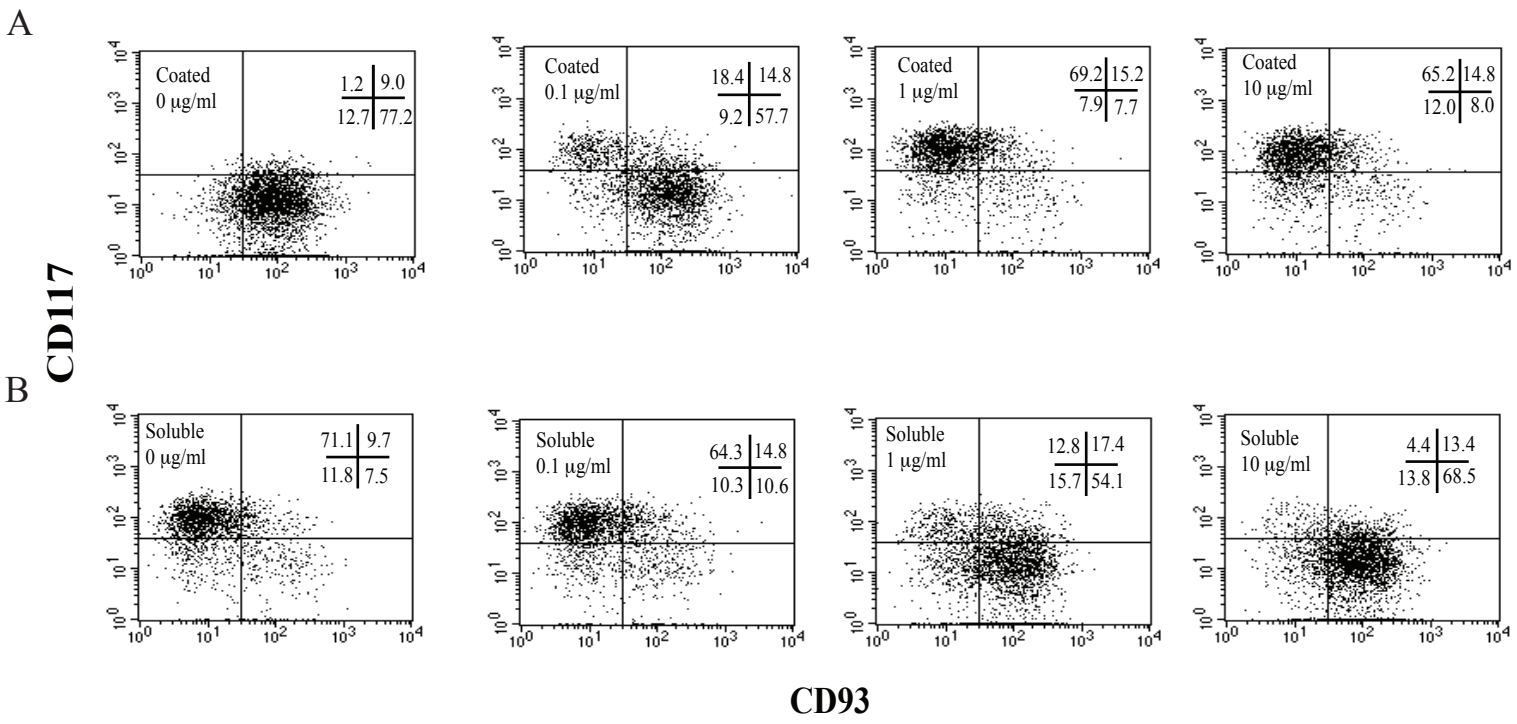

Fig. S2. *Plate-bound DLL4-Fc provokes de-differentiation of pro B cells and induces T cell differentiation in a doses dependent fashion*.

(A) Different amounts of DLL4-Fc fusion protein were added to 10 µg/ml plastic bound monoclonal anti-human IgG1Fc (Huf5.4) antibody and $1x10^6$ $Pax5^{-/-}$ pro-B cells cultured for 5 days in the presence of IL-7 and SCF. Dot plots show cell surface expression of CD117 (c-kit) and CD93. Up-regulation of CD117 accompanied by down-modulation of CD93 marks development in the direction of T cells. (B) Notch signaling was blocked using increasing amounts of soluble DLL4-Fc added to the medium of $Pax5^{-/-}$ pro-B cells cultured on 1µg/ml plate-bound DLL4-Fc in the presence of IL-7 and SCF.

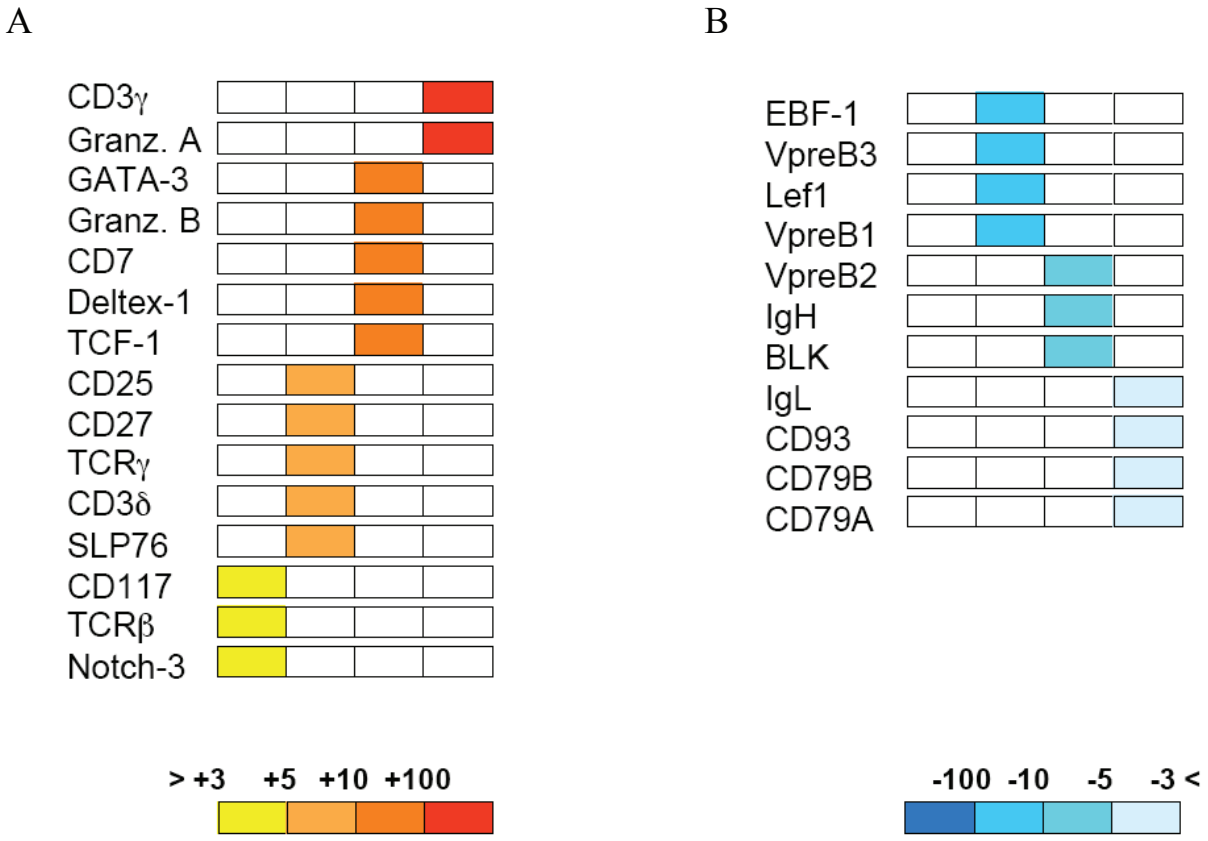

Fig. S3. *Gene expression pattern of a Pax5$^{-/-}$ pro-B cell line in its response to Notch signaling*.
Cells were collected on day 5 after culture on plate-bound DLL4 and sorted to isolate RNA from a homogenous population, which was analyzed on Affymetrix Mouse Genome 1.0 ST gene array. Fold increased or decreased expression of selected T-cell (A) or B-cell (B) specific genes are indicated in comparison to non-induced Pax5$^{-/-}$ pro-B cells.

III

Full-Length article

The amount of self-antigen determines the effector function of murine T cells escaping negative selection.

Running title: Low dose of neo-self-antigen promotes Treg development

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Abbreviations

6.5^{+}, cells positively stained by the rat anti-TCR HA clonotype specific mAb 6.5

ANA, anti-nuclear auto-antibodies

CD11c HA^{high}, mouse expressing a membrane bound form of HA under control of the CD11c promoter at a high level

CD11c HA^{low}, mouse expressing a membrane bound form of HA under control of the CD11c promoter at a low level

CD4sp, CD4 single positive

CD62L, CD62 ligand

CD8sp, CD8 single positive

DC, dendritic cell

F1 HA^{high}, F1 double transgenic mouse of the breeding TCR HA x CD11c HA^{high}

F1 HA^{low}, F1 double transgenic mouse of the breeding TCR HA x CD11c HA^{low}

HA, hemagglutinin protein from the PR8 influenza virus strain

Igκ HA, mouse expressing a membrane bound form of HA under control of the Ig kappa promoter

TCR HA, mouse expressing I-E^{d} restricted, HA-specific transgenic $\alpha\beta$ T cell receptor

TEC, thymic epithelial cell

Treg, regulatory T cell

TSA, tissue-specific antigen

Summary

Autoimmune diseases develop when self-specific T cells that escaped negative selection initiate a harmful immune response against self. However, factors, which influence the initiation and progression of an autoimmune response, remain incompletely understood. By establishing a double transgenic mouse system in which different amounts of a cell-surface neo-self antigen are expressed under the CD11c promoter, we demonstrate that antigen dose dramatically influences T cell tolerance mechanisms. Moderate antigen expression favors the development of antigen-specific regulatory T cells and the establishment of a tolerogenic environment. In marked contrast, high dose of antigen expression results in very stringent negative selection, in poor development of antigen-specific regulatory T cells and the early onset of anemia and splenomegaly and the late development of arthritis and high titers of IgG auto-antibodies. Disease is initiated by autoreactive T cells, which escape negative selection by expressing a second T cell receptor with a different specificity or an altered affinity. Transfer of antigen-specific regulatory T cells ameliorates the early onset signs of disease but does not prevent the development of long-term chronic pathologies.

Introduction

The rearrangement of T cell receptor (TCR) α- and β-chain loci is a random process and therefore can result in the development of T cells expressing an autoreactive TCR. Under physiological conditions these T cells are deleted by a process called negative selection [1-5]. However, as various model systems have indicated, the development into $Foxp3^+$ regulatory CD4 T cells (Treg) is an alternative fate for autoreactive T cells in the thymus [6-11]. Nevertheless, despite negative selection and/or Treg induction, mature self-specific T cells may be found in the periphery due to e.g. weak or absence of tissue-specific antigen (TSA) expression in the thymus. These T cells are prevented from causing autoimmunity by mechanisms such as anergy [12, 13] or control by regulatory T cells [6-11]. Self-specific mature T cells may differentiate into Foxp3 positive or negative regulatory T cells under conditions that involve low antigenic stimulation in absence of co-stimulation or antigen presentation by particular subsets of antigen presenting cells [14-21].

Autoimmune diseases develop when one or several mechanisms of T cell tolerance fail. In mice and man, Foxp3 deficiency leads to a very severe disease due to complete absence of $Foxp3^+$ regulatory T cells [22, 23]. Aire is a transcription factor that contributes to expression of TSA in the thymus [24, 25]. Aire deficiency causes a disease that affects many organs and glands likely due to absence of negative selection of TSA-specific T cells [24, 25]. Similarly, in NOD mice, β-cell destruction is caused by cells that escape negative selection [26].

It has been shown, both in mice and humans, that up to one third of mature T cells express two different TCR α-chains [27-32]. This suggests, that self-specific T cells may escape negative selection by expressing a second non-self specific TCR during

development and give rise to autoimmunity. A study by Sarukhan et al [28] strongly supports such a scenario.

In the present study, we describe two transgenic mouse lines that express different amounts of influenza virus hemagglutinin (HA) under the CD11c promoter. By crossing these mice with transgenic mice expressing an MHC class II restricted HA-specific TCR (called TCR HA hereafter), we investigated the role of antigen dose in immune tolerance induction. Our findings clearly demonstrate that the antigenic dose dramatically influences T cell tolerance. The amount of antigen expressed by DC's and TECs in the thymus does seem to influence the stringency of negative selection. Thus, low HA expression correlates with the induction of Treg development, whereas high HA expression correlates with a very stringent negative selection process and poor regulatory T cell development, increased T cell activation and development of autoimmune disease.

Results

Mouse model

To target the expression of HA protein to dendritic cells, we generated transgenic mice expressing a membrane bound form of HA under control of the CD11c promoter. Two different transgenic mouse lines were obtained. As shown in Figure 1A (left panels), HA expression was readily detectable on CD11c bright splenocytes from both of the HA transgenic lines but not WT BALB/c mice. However, the amount of HA protein expressed by $CD11c^{+}MHCII^{+}$ cells from the spleen and the thymus was dramatically different between the two transgenic lines named hereafter "CD11c HA^{low}" and "CD11c HA^{high}" respectively (Figure 1A, middle and lower panels). Differences in HA expression might be explained by differences in copy numbers of the transgene integrated. Southern blot analysis indeed revealed an at least 10 fold higher HA copy number in CD11c HA^{high} than in CD11c HA^{low} mice (Figure 1B). In order to verify that HA expression correlated with peptide presentation by MHC class II molecules, we addressed the ability of sorted dendritic cells from the different CD11c HA transgenic mice or wild type mice to induce the proliferation of $CD4^{+}$ T cells from TCR HA mice which express an I-E^{d} restricted, HA-specific transgenic T cell receptor [33]. As shown in Figure 1C, at a ratio of 10 to 1 (CD4 T cells : DC) dendritic cells from both of the CD11c HA transgenic mice induced robust proliferation of HA specific CD4 T cells compared to dendritic cells from wild-type mice, which are non-stimulatory (Figure 1C, upper panels). At a ratio of 90:1 (CD4:DC), dendritic cells from CD11c HA^{high} transgenic mice induce T cell proliferation as efficiently as dendritic cells from CD11c HA^{low} transgenic mice at a ratio of 10:1. Thus, dendritic cells from CD11c HA^{high} transgenic mice are about ten

fold more potent in their ability to induce HA-specific CD4 T cell proliferation as compared to dendritic cells from CD11c HA^{low} mice due to increased HA presentation by surface expressed MHC class II on dendritic cells. The expression level of MHC II, CD80, CD86 and CD40 was very similar between dendritic cells from the spleen of WT, CD11c HA^{low} or CD11c HA^{high} transgenic mice (Supplemental Figure 1). Therefore, HA expression per se does not change the maturation status of dendritic cells as monitored by expression of costimulatory molecules. HA expression under the CD11c promoter was not tightly restricted to the DC compartment. By qPCR, it could be shown that HA is expressed strongly by cDC's of the CD11c HA^{high} mouse strain. NK cells, as well, were found to express HA on a high level in CD11c HA^{high} mice. T and B lymphocytes of the same strain showed a very weak amplification signal. Expression of the HA transgene in the CD11c HA^{low} mouse strain was lower in all subsets. Only in myeloid cells of the CD11c HA^{high} and of the CD11c HA^{low} mouse strain, as well as in all cell samples derived from WT BALB/c mice, HA expression could not be detected (Figure 1D). Thus, HA expression is not only restricted to the DC compartment. Thymic epithelial cells (TEC) were shown to express the HA antigen as well. However, for detection of HA expression in TECs a more sensitive approach was chosen and results are shown and will be discussed referring to the chimaera data shown in Figure 3 and 5.

Autoimmune disease develops in TCR HA x CD11c HA^{high} mice but not in TCR HA x CD11c HA^{low} mice

In order to investigate the role of dendritic cells in immune tolerance we generated (TCR HA x CD11c HA^{low})F1 mice (called F1 HA^{low} hereafter) and (TCR HA x CD11c HA^{high})F1 mice (called F1 HA^{high} hereafter) and monitored their health status. All young (< 4 weeks old) F1 HA^{high} mice suffered from severe anemia as determined

by hematocrit levels (33.5±3.0 versus 42.5±2.6 in TCR HA mice) and showed splenomegaly, which to a large extent was due to an accumulation of myeloid cells (16.5±6.4x10^6 versus 0.8±0.4 x10^6 in TCR HA mice) (Figure 2, A and C). In sharp contrast, hematocrit levels and myeloid cell numbers in the spleen of F1 HA^{low} mice were comparable to what was found in TCR HA mice (Figure 2, A and C). Moreover, histological analysis revealed a completely disturbed organization of the T and B cell zones in the spleens of young F1 HA^{high} mice whereas these were normal in F1 HA^{low} mice of the same age (Figure 2, E and F). No germinal centers were detectable in the spleens of young F1 HA^{high} or F1 HA^{low} mice (Figure 2E).

In marked contrast hematocrit levels in adult F1 HA^{high} mice (> 8 weeks old) were normalized (46.8± 1.9 versus 48.3±1.0 in TCR HA mice) (Figure 2B). Moreover, decreased but still significant myeloid cell accumulation was observed in the spleens of adult F1 HA^{high} mice (11.0± 5.9 x10^6 versus 3.1±0.8 x10^6 in TCR HA mice) (Figure 2D). In adult F1 HA^{high} but not F1 HA^{low} mice high titers of IgG anti-nuclear auto-antibodies (ANA) were detected in the blood (Figure 2G). Unlike in young, adult F1 HA^{high} mice showed a normal T and B cell zone organization in the spleen (Figure 2F). Moreover, high numbers of germinal centers were detectable in the spleen of F1 HA^{high} but not in F1 HA^{low} mice (Figure 2E). All adult F1 HA^{high} mice showed with time a thickening of their paws. Histological examination of the joints revealed a severe arthritis characterized by mainly acute inflammation with massive swelling, cellular infiltration of the synovial membrane and focal destruction of the cartilage surface; polymorphonuclear granulocytes along with fibroblasts and histiocytes were the major component of the cellular infiltrate (Figure 2I and J). In the joints of the control mice no histological abnormalities were observed (Figure 2H).

Altogether, young F1 HA^{high} mice suffer from severe anemia, display a massive accumulation of myeloid cells in the spleen and show a complete disturbed T and B cell zone architecture in their secondary lymphoid organs during the first weeks after birth. On the other hand, in adult F1 HA^{high} mice anemia faded away and T and B cell zone architecture in their secondary lymphoid organs was normalized. However, these adult mice all developed arthritis and had high titers of IgG ANA. In sharp contrast, the phenotype of F1 HA^{low} mice was similar to the phenotype of TCR HA single transgenic mice suggesting that F1 HA^{low} mice did not suffer from any disease.

Central tolerance in F1 HA^{low} and F1 HA^{high} mice

The finding that F1 HA^{high} mice developed various types of autoimmune like diseases strongly suggested that the induction of HA specific T cell tolerance was impaired in these mice. Therefore we investigated the induction of central tolerance by monitoring the fate of thymocytes encountering their cognate antigen in F1 HA^{low} or F1 HA^{high} as opposed to TCR HA and WT mice. In the thymus of TCR HA mice, approximately 40% of CD4 single positive (CD4sp) cells express the HA-specific transgenic T cell receptor as indicated by the binding of the idiotype specific 6.5 mAb that recognizes only the combination of transgenic Vβ8 and Vα4 (idiotype specific) (Figure 3A, upper panel). The rest of CD4sp cells expressed the transgenic Vβ8-chain and, based on being negative for 6.5, an endogenous rearranged α-chain (Figure 3A, upper panel to the right). When HA antigen was expressed a dramatic negative selection of HA-specific 6.5^{+} CD4sp cells occurred regardless of the dose of antigen expressed (Figure 3A, upper and lower middle panels). All CD4sp cells from F1 HA^{low} mice did express Vβ8, whereas about 20% of the CD4sp in the thymus of F1 HA^{high} were Vβ8 low/negative, suggesting an even stronger negative selection due to higher expression of HA. Importantly, despite drastic deletion of HA-specific CD4sp

T cells in F1 HA^{low} and F1 HA^{high}, 2-5% of the CD4sp cells were found to be 6.5^+ and therefore expressed the transgenic TCR (Figure 3A, middle panels). No 6.5^+ CD4sp were detectable in WT mice (Figure 3A, lower panel).

The development of low numbers of 6.5^+ CD4sp cells in both F1 HA^{low} and F1 HA^{high} suggests that these cells escaped negative selection. To investigate whether these cells underwent positive and negative selection while expressing solely the HA-specific T cell receptor or whether cells could develop by expressing an endogenously rearranged receptor, we bred F1 HA^{low} and F1 HA^{high} mice onto a *Rag-2* deficient genetic background. In TCR HA transgenic *Rag-2* deficient mice, all CD4sp cells were 6.5^+ and almost no CD8sp could be found (Figure 3B, upper panel). In marked contrast, both F1 HA^{low} and F1 HA^{high} mice on a *Rag-2*$^{-/-}$ genetic background showed an arrest of thymocyte development at the double positive stage (Figure 3B, middle and lower panel). Moreover, no T cells could be found in the periphery of these animals (data not shown). This finding demonstrates, that the development of CD4sp cells in F1 HA^{low} and F1 HA^{high} mice on a rearrangement sufficient genetic background, requires the expression of another TCR with no or only low affinity for HA.

Peripheral tolerance in F1 HA^{low} and F1 HA^{high} mice

In the next set of experiments we analyzed the peripheral CD4 T cell compartment in F1 HA^{low} and F1 HA^{high} mice and compared this to TCR HA and WT mice. In the peripheral lymphoid organs of both F1 HA^{low} and F1 HA^{high} mice around 3 – 5% of CD4 T cells express the transgenic TCR as indicated by being 6.5^+. In TCR HA mice the percentage of CD4 cells expressing 6.5 varied from 20 – 40%, whereas no 6.5^+ cells were detectable in WT mice (Figure 4A). Since T cell development in F1 HA^{low} and F1 HA^{high} mice on a *Rag-2* deficient background is blocked at the double positive

stage in the thymus (Figure 3B) it was likely that 6.5^+ CD4 T cells in F1 HA^{low} and F1 HA^{high} mice express yet another non-transgenic TCR or non-transgenic TCR component. The TCR α-chain of the HA specific TCR uses a Vα4 variable region. The fact that 10 – 20% of the 6.5^+ CD4 cells in F1 HA^{low} and F1 HA^{high} mice expressed a TCR α-chain using a Vα2 variable region strongly supported this assumption (Figure 4B, middle and right panel). To our surprise, about 25% of the 6.5^+ CD4 T cells in TCR HA mice also expressed a TCR α-chain using a Vα2 variable region (Figure 4B, left panel). One possible reason for this might be that T cells expressing only the HA specific TCR are poorly positively selected.

In order to determine the activation status of CD4 T cells in the various mice we analyzed their CD44, CD62L and CD69 surface expression patterns. The vast majority (87%) of the 6.5^+ CD4 T cells in TCR HA mice had a naïve phenotype ($CD62L^+$ and $CD44^{intermediate/low}$) and only around 10% had an activated/memory phenotype ($CD62L^-$ and $CD44^{high}$) and around 30% of these expressed the early activation marker CD69 (Figure 4, C and D, left panels). More than half (56.2%) of the 6.5^+ CD4 T cells in F1 HA^{low} mice had a naïve phenotype ($CD62L^+$ and $CD44^{intermediate/low}$) whereas around 40% had an activated/memory phenotype ($CD62L^-$ and $CD44^{high}$). Of these around 40% expressed the early activation marker CD69. In marked contrast, only 15% of the 6.5^+ CD4 T cells in F1 HA^{high} mice had a naïve phenotype and over 80% had an activated/memory phenotype. Of these 70% expressed CD69 (Figure 4, C and D, middle and right panels). Thus, the relative number of activated 6.5^+ CD4 T cells in F1 HA^{low} mice was about 3 fold higher than in TCR HA mice whereas in F1 HA^{high} mice practically all 6.5^+ CD4 T cells were found to be activated. Since the 6.5^+ CD4 T cell population comprises only a small part of the total CD4 compartment in F1 HA^{low} and F1 HA^{high} mice, we also analyzed

the activation status of the total CD4 T cells in the different mice. The CD44, CD62L and CD69 expression patterns of CD4 T cells from WT, TCR HA and F1 HA^{low} mice were practically identical in that over 75% of the cells had a naïve phenotype ($CD62L^{+}$ and $CD44^{intermediate/low}$) whereas around 20% had an activated/memory phenotype ($CD62L^{-}$ and $CD44^{high}$) of which around 30% expressed CD69 (Supplemental Figure 2, A and B). In marked contrast only 32% of the CD4 T cells in F1 HA^{high} mice showed a naïve phenotype whereas more than 65% had an activated/memory phenotype of which around half expressed CD69 (Supplemental Figure 2, A and B). This latter finding suggests that 6.5^{-} CD4 T cells are HA reactive and get activated in F1 HA^{high} mice. The fact, that about 30% of the 6.5^{-} CD4 T cells from TCR HA mice showed a proliferative response, when stimulated with HA expressing dendritic cells, supports this hypothesis (Supplemental Figure 2C).

We also determined the percentage of CD4 cells producing GM-CSF, IFN-γ, IL-4 and IL-17 in young and adult TCR HA, F1 HA^{low} and F1 HA^{high} mice. In none of the mice significant numbers of IL-4 and IL-17 producing CD4 T cells were detected (data not shown). Moreover, in young and adult TCR HA and F1 HA^{low} mice also no significant numbers of GM-CSF and IFN-γ producing CD4 T cells were detectable (Figure 4, E-H). In marked contrast, in young F1 HA^{high} mice around 5% (5.2 ± 1.7%) CD4 T cells produced GM-CSF and around 23% (23.6 ± 3.7%) produced IFN-γ (Figure 4, E and G). In adult F1 HA^{high} mice the percentage of CD4 cells producing GM-CSF or IFN-γ decreased but was however still significantly higher than the percentages found in adult TCR HA and F1 HA^{low} mice (Figure 4, F and H).

Overall, our findings show that CD4 T cells in F1 HA^{high} mice are activated and that relative high numbers of cells produce GM-CSF and IFN-γ likely causing

pathologies observed in F1 HA^{high} mice. No autoimmunity and no or only poor CD4 T cell activation was observed in TCR HA and F1 HA^{low} mice.

B cells from CD11c HA^{high} mice efficiently stimulate 6.5^+ T cells

The strong B cell activation observed in F1 HA^{high} mice suggests to underly a cognate interaction between HA specific T cells and HA peptide-presenting B cells. Therefore we analyzed whether CD11c expressing B cells could be found in the spleens of CD11cHA^{low}, CD11cHA^{high} and WT BALB/c mice and whether a correlation with HA expression existed. About 1.5% of the cells in the spleens of these mice expressed CD11c and CD19 (Supplemental Figure 3). Moreover, in CD11c HA^{high} mice the majority of these expressed HA although about one order of magnitude lower than dendritic cells ($CD11c^{bright}$ $CD19^-$). A low level of surface HA expression was detectable on CD11c negative B cells (Supplemental Figure 3). No HA expression was detectable on $CD11c^+CD19^+$ or $CD11c^-CD19^+$ B cells derived from CD11c HA^{low} or BALB/c mice. The $CD11c^+CD19^+$ were bona fide B cells since they expressed IgM, IgD, CD21 and CD23 (Supplemental Figure 4). In fact the $CD11c^+CD19^+$ cells seemed to be enriched for marginal zone B cells since the percentage of $IgM^{high}IgD^{low}$ cells and the percentage of $CD21^{high}CD23^{low}$ cells was 2 – 4 fold higher than in the total splenic CD19 population (Supplemental Figure 4).

In order to test the capacity of the $CD11c^+$ and $CD11c^-$ B cells to stimulate HA specific T cells $CD11c^+CD19^+$ and $CD11c^-CD19^+$ cells were sorted and cultured with lymph node cells from TCR HA mice. $CD11c^{bright}CD19^-$ dendritic cells were used as controls. As expected no proliferation was observed using dendritic cells, $CD11c^+$ or $CD11c^-$ B cells from wt BALB/c mice as stimulator cells (Supplemental Figure 5). On the other hand dendritic cells from both CD11c HA^{low} and CD11c HA^{high} mice induced a strong proliferation of T cells derived from TCR HA mice (Supplemental

Figure 5). No or only marginal proliferation was observed when $CD11c^+$ and $CD11c^-$ B cells from CD11c HA^{low} mice were used as APC's (Supplemental Figure 5). In marked contrast, $CD11c^+$ B cells from CD11c HA^{high} mice induced a strong proliferative response whereas $CD11c^-$ B cells from these mice induced a weaker, but still significant, proliferative response. Therefore, the B cell activation observed in F1 HA^{high} mice is likely to be caused by the cognate interaction of HA specific transgenic T cells and B cells expressing HA as well as B cells that might have picked up HA.

Increased frequency of HA specific regulatory T cells in the thymus and periphery of F1 HA^{low} mice

In order to determine the number of 6.5^+ CD4sp Treg cells in the thymus of the various mice, we tested Foxp3 expression. In young and adult TCR HA mice only around 1% of the 6.5^+ CD4sp cells expressed Foxp3 (young: 1.0 ± 0.1% and adult: 0.6 ± 0.2%) (Figure 5, A and B). Thus, in the absence of HA, T cells specific for HA very poorly develop into Treg. In young F1 HA^{low} mice around 15% (16.2 ± 9.2%) and in adult F1 HA^{low} mice around 10% (9.0 ± 0.9%) of the 6.5^+ CD4sp expressed Foxp3. In marked contrast, in the thymus of young F1 HA^{high} mice only 1.5% (1.3 ± 0.3%) of the 6.5^+ CD4sp expressed Foxp3 whereas around 4% (3.7 ± 1.1%) of the 6.5^+ CD4sp expressed Foxp3 in adult F1 HA^{high} mice (Figure 5, A and B). These findings indicate that although antigen expression by dendritic cells favors negative selection, low level of antigen expression correlates with increased development of antigen-specific Treg as compared with high antigen expression.

The relatively high frequency of HA specific Treg's in the thymus of F1 HA^{low} might suggest that the apparent absence of $CD4^+$ T cell activation in F1 HA^{low} mice might be due to an increased frequency of 6.5^+ regulatory T cells in the periphery. Therefore we determined the proportion of $Foxp3^+$ cells among the 6.5^+ $CD4^+$ cells in

the spleen of TCR HA, F1 HA^{low} and F1 HA^{high} transgenic mice. As shown in Figure 5, C and D, about 34% (34.0 ± 7.2%) and 18% (18.4 ± 2.1%) of the 6.5^+ $CD4^+$ T cells expressed Foxp3 in young and adult F1 HA^{low} mice respectively. In sharp contrast, practically no 6.5^+ $CD4^+$ T cells expressing Foxp3 were detectable in young F1 HA^{high} mice (2.9 ± 1.1%). In adult F1 HA^{high} mice the frequencies of 6.5^+ Treg cells was still very low but significantly higher than in their young counterparts (6.1 ± 1.6% vs 2.9 ± 1.1%). As expected, 6.5^+ T cells generated in the absence of the antigen HA (TCR HA mice) only very poorly develop into Foxp3 positive cells. Thus, low expression of HA correlates with increased ratio of antigen-specific Treg and establishment of tolerance whereas high antigen expression favors effector T cell differentiation over Treg development causing autoimmunity.

HA transgene expression in non-hematopoietic cells and its contribution to thymic selection

Since HA expression under the CD11c promoter was not restricted to DC's only, we tested whether HA is expressed in the non-hematopoetic compartment as well. TEC are non-hematopoietic antigen-presenting cells in the thymus and were shown to play an important role in Treg development. For effective thymic selection processes already very low levels of presented antigen can be sufficient. To reveal the low antigen expression level of TEC's, BM chimaeras were generated to use the very sensitive thymic selection processes as read-out. Lethally irradiated CD11c HA^{high}, CD11c HA^{low} and Igκ HA mice were reconstituted with bone marrow derived from TCR HA mice. In Igκ HA mice the HA antigen is expressed under control of the κ light chain promoter leading to HA expression by TEC's and B cells [34]. Expression of the transgenic TCR HA within the CD4 T cell compartment of thymus and spleen was analyzed after 6 weeks (Figure 6A). In addition BM chimaeras were analyzed for

expression of the transcription factor FoxP3 (Figure 6B and C). In all hosts TCR HA expressing CD4 T cells could be positively selected. However, percentages of 6.5^+ CD4 single-positive T cells differed among the different hosts in the thymus (Figure 6A). In CD11c HA^{high} BM chimaeras only 4.4±0.5% of the single-positive CD4 T cells expressed the TCR HA, whereas in CD11c HA^{low} BM chimaeras about 18.9±4.9% 6.5^+ CD4 single-positive T cells were detected. In BALB/c and Igκ HA BM chimaeras, 43.2±5.2% and 9.5±2.1% 6.5^+ CD4 single-positve T cells were found respectively. In the periphery slightly lower percentages for 6.5^+ CD4 T cells were observed compared to levels detected in the thymus (CD11c HA^{high}: 5.0±0.2%, CD11c HA^{low}: 11.1±3.7%, BALB/c: 25.5±3.1%, Igκ HA: 5.7±0.8%). According to these results the HA transgene under the CD11c promoter is expressed in the TEC compartment and TECs could be shown to efficiently contribute to positive and negative selection processes independently of transgene expression in the lymphoid compartment.

To reveal the contribution of the TEC compartment to the development of 6.5^+ Treg's, FoxP3 expression was detected by intracellular staining in BM chimaeras (as described before) 6 weeks after reconstitution. Dramatic differences in the 6.5^+ Treg compartment were found comparing the different hosts, namely CD11c HA^{high}, CD11c HA^{low}, Igκ HA and BALB/c. In absolute numbers only about 100 6.5^+ Treg's were detected in the thymus of CD11c HA^{high} BM chimaeras, whereas in CD11c HA^{low} BM chimaeras $3.3*10^4±3.0*10^4$ 6.5^+ Treg's were found. BALB/c mice generated $1.5*10^4±1.0*10^4$ and Igκ HA mice $1.9*10^3±1.2*10^3$ 6.5^+ Treg's. In the periphery total numbers of 6.5^+ Treg's were increased in all hosts compared to thymus. However, in CD11c HA^{high} BM chimaeras the 6.5^+ Treg population ($4.5*10^3±1.6*10^3$) still was markedly smaller compared to the other hosts (CD11c

HA^{low}: $4.2*10^4 \pm 2.1*10^4$, BALB/c: $7.2*10^4 \pm 3.4*10^4$, Igκ HA: $1.8*10^4 \pm 0.6*10^4$). Considering the size of the complete CD4 T cell compartment, only CD11c HA^{low} and Igκ HA BM chimaeras showed with 32.2±4.1% and 17.8±1.9% an increased generation of 6.5^+ Treg's compared to BALB/c controls (1.0±0.2%) and CD11c HA^{high} BM chimaeras (3.8±0.6%) in the thymus (Figure 6B). In the periphery similar results were found for 6.5^+ Treg's comparing CD11c HA^{low} (39.9±3.5%), Igκ HA (58.3±5.1%), BALB/c (8.5±2.1) and CD11c HA^{high} (13.7±3.3%) BM chimaeras (Figure 6C). Thus, TECs could not only be shown to efficiently contribute to positive and negative selection processes but as well to Treg development in systems featuring a low antigen expression level.

Transfer of HA specific Treg's can in part inhibit the development of autoimmunity in F1 HA^{high} mice

The findings described above suggested that the autoimmunity observed in F1 HA^{high} mice was due to an inefficient development of a HA specific Treg compartment. In order to test this hypothesis, we sorted HA-specific regulatory T cells from TCR HA x Igκ HA double transgenic mice that were described to generate high percentages of HA specific Treg's [8] as well as polyclonal Treg's from WT BALB/c mice and transferred them into newborn F1 HA^{high} mice. As summarized in Figure 7A, the transfer of 6.5^+ Treg's (HA-Treg's) but not polyclonal Treg's (WT Treg's) prevented the onset of anemia in young F1 HA^{high} mice. Moreover, the frequency of IFN-γ and GM-CSF producing CD4 T cells reduced by a factor of 2 upon transfer of antigen specific Treg's but not by polyclonal Treg's (Figure 7, B and C). These results suggest that the signs of autoimmunity observed in young F1 HA^{high} mice is due to an absence of HA specific Treg's. However, the onset of arthritis and the development of

IgG auto-antibodies in adult F1 HA^{high} mice could not be inhibited by HA-specific Treg's (data not shown).

Discussion

Here, using transgenic mice expressing HA under control of the CD11c promoter we studied the influence of the dose of antigen presented by DC's on central and peripheral T cell tolerance and the development of autoimmunity. CD11c is widely regarded as a dendritic cell marker [35, 36]. However, low levels of CD11c are also expressed by NK cells [37] and B cells expressing CD11c have also been described [38, 39]. In our system, expression of the neo-self-antigen HA under the CD11c promoter was not only directed to DC's. In the CD11c HA^{high} mouse strain, also NK cells were found to express HA. The highest HA expression level in the CD11c HA^{low} strain was as well found in the DC compartment. However, the HA transgene expression pattern in CD11c HA^{low} mice differed from the one observed in CD11c HA^{high} mice, probably due to different transgene insertion sites. Results derived from BM chimaeras indicate that the HA transgene is as well expressed by TEC's.

Our findings show, that independent of the dose of the neo-self-antigen HA expressed in the thymus and of mouse age, negative selection of HA-specific thymocytes is efficient since DC's from both F1 HA^{low} and F1 HA^{high} mice were equally able to delete 6.5^{+} (mAb specific for TCR HA) thymocytes and negative selection was complete in both systems, on a *Rag-2* deficient background. However, we observed interesting differences in the negative selection process in F1 HA^{low} and F1 HA^{high} mice. All CD4sp thymocytes in F1 HA^{low}, as in TCR HA mice, expressed high levels of the transgenic Vβ8 chain (Figure 3), indicating that in F1 HA^{low} mice TCR β-chain allelic exclusion is very efficient. In contrast, in F1 HA^{high} mice, about 20% of the CD4sp cells expressed only low or not detectable levels of Vβ8. This finding suggests, that negative selection in F1 HA^{high} mice is more stringent and therefore CD4sp T cells, that escaped negative selection by not obeying the rule of

TCR β-chain allelic exclusion, are proportionally enriched. In both, F1 HA^{low} and F1 HA^{high} mice, on a *Rag-2* deficient background, negative selection is complete. Nevertheless, the finding that double positive thymocytes from *Rag-2* deficient F1 HA^{low} mice express low levels of transgenic TCR on their surface whereas double positive thymocytes from *Rag-2* deficient F1 HA^{high} mice are 6.5 negative (Figure 3), also suggests that in the case where DC's express higher amounts of antigen negative selection is more stringent. Thus, negative selection seems to be determined by the affinity and avidity of the interaction between TCR and its corresponding antigen.

The finding that mature T cells are absent in F1 HA^{low} and F1 HA^{high} mice on a *Rag-2* deficient background demonstrates that in *Rag-2* proficient mice, T cells can only escape negative selection by expression of a second non-HA specific TCR or of a TCR with an altered affinity to the antigen HA. The expression of a non-transgenic TCR α-chain by a proportion of mature CD4 cells in F1 HA^{low} and F1 HA^{high} mice supports this hypothesis. As already suggested by others [40], the rearrangement of an endogenous α-chain is probably occurring in a stochastic manner prior to negative selection. In other words, it is likely that T cells expressing an endogenous rearranged TCR α-chain with a pairing efficiency to the transgenic TCR β-chain higher than the one of the transgenic α-chain will not or poorly express the transgenic HA-specific TCR and therefore could escape negative selection. It is important to note that under physiological conditions, significant numbers of T cells expressing 2 different α-chains have been found both in human blood and the periphery of WT mice [27-32]. Moreover, it has been indicated that T cells expressing two TCR α-chains can induce autoimmunity [28].

In the periphery of F1 HA^{low} and F1 HA^{high} mice CD4 T cells with a HA specific TCR could be found. Based on the fact that no peripheral T cells were detectable in F1

HA^{low} and F1 HA^{high} mice on a *Rag-2* deficient background these T cells must have escaped negative selection by the expression of a second TCR in both double transgenic mouse strains. However, the phenotype of the overall CD4 T cell population, including the 6.5^{+} ones, in the spleen of F1 HA^{low} and F1 HA^{high} mice differed dramatically. Unlike F1 HA^{low} CD4 cells, most of the splenic CD4 cells in F1 HA^{high} mice had an activated phenotype and, especially in young mice, a high number of them produced IFN-γ and GM-CSF. Disease phenotype is rather unlikely to be caused by a strong T cell proliferation due to lymphopenia, that could be caused by extensive negative selection in F1 CD11c HA^{high} mice. Lymphopenia induced proliferation was shown to correlate with a memory-phenotype T cell compartment [41].

It has been demonstrated that TCR stimulation in absence of co-stimulation leads to T cell tolerance by deletion, anergy or Treg conversion mechanisms. It is therefore surprising that T cells get activated in the first place since dendritic cells from CD11c HA^{high} mice are not more activated than dendritic cells from CD11c HA^{low} or WT mice. We would argue, that even in the absence of infectious agents, it is very likely that few dendritic cells get activated due to tissue damage, contact with commensal bacteria or local inflammation. The chance that any HA-specific T cells encounter one of the few activated DC's is considerable and cumulative chances over time probably reach 100%. Once a T cell gets activated, it will express CD40L and be able to activate dendritic cells through CD40-CD40L interaction, newly activated dendritic cells will activate more T cells therefore creating an activation loop that never stops.

Differences in T cell activation go in parallel with the presence or absence of Treg cells. A remarkable difference between CD4sp T cells of F1 HA^{low} and F1 HA^{high}

mice is the Foxp3 expression in the thymus. Around 10 – 20% of 6.5^+ CD4sp cells of F1 HA^{low} mice express Foxp3, whereas only very few of them of F1 HA^{high} mice do express Foxp3 in absolute numbers.

TEC's were described to be able to induce Treg development [42]. To study if HA expression under the CD11c promoter leads to HA expression by TEC's CD11c HA^{high}, CD11c HA^{low} and Igκ HA mice were lethally irradiated and reconstituted with bone marrow derived from TCR HA mice. In CD11c HA^{low} as well as in Igκ HA BM chimaeras 32.2±4.1% and 17.8±1.8% 6.5^+ Treg's which corresponds to about 33000 and 2000 cells in absolute numbers, were generated respectively. These results suggest that the HA antigen is not only expressed by DC's as thymic APC but as well by TEC's. In contrast, in CD11c HA^{high} BM chimaeras only 3.8±0.6% 6.5^+ Treg's were detected. In absolute numbers that corresponds to about 100 6.5^+ Treg cells only. Thus, the avidity of the TCR – thymic APC interaction seems to control not only the stringency of negative selection but as well Treg formation i.e. for a strong TCR self-affinity, low avidity allows Treg development whereas high avidity seems to contribute to a very stringent negative selection process. Very recently, it was shown that not only TEC's but as well a subset of the DC compartment was able to support Treg development by rescue of developing Treg cells from apoptosis via CD27-CD70 pathway [43]. These findings suggest, that in our HA mouse model several APC's contribute to thymic selection and that avidity seems to be a superior factor for these selection processes. In a recent study by Hinterberger *et al*, a similar conclusion was drawn based on the findings, that Treg development in the thymus increased upon decrease of MHC class II expression [44]. Moreover, in this context, it is noteworthy, that conversion of conventional T cells into Treg's in the periphery can be achieved by targeting low but not high concentrations of antigen to DC's [19, 20, 45].

The exact mechanism for Treg development is not yet understood. One plausible explanation for Treg generation in our system would be the selection into Treg lineage by usage of a second endogenously rearranged TCR. In this case, Treg's would be selected upon interaction with an unknown endogenous antigen. Only in the periphery these Treg's could reexpress the transgenic TCR on the surface and mediate Treg function. However, assuming Treg selection is based on a second endogenously rearranged TCR, we have no explanation for differences in total thymic Treg occurrence comparing F1 CD11c HA^{low} and F1 CD11c HA^{high} as well as CD11c HA^{low} and CD11c HA^{high} TCR HA BM chimaeras. In case Treg selection is based on a second TCR with unknown specificity, one would expect a comparable amount of total thymic Treg's as the selecting ligands would be expected to be expressed on comparable levels. However, one could argue that negative selection is more stringent in CD11c HA^{high} background as deletion of thymocytes affects as well thymocytes expressing the transgenic Vβ8 in combination with an endogenous Vα chain. In CD11c HA^{low} background thymocytes are almost all positive for vβ8 and therefore it is likely that in such a system more T cells expressing 2 TCRs are generated. Thus, Treg numbers could be increased in general. Several groups provided data supporting an instructive Treg model [46, 47]. According to this model the number of selected Treg cells is inversely proportional to the fraction of thymocytes expressing transgenic TCR. This finding was interpreted as evidence that Treg precursors need an intense interaction with a selecting ligand which is achieved by "diluting" Treg precursors. In our model, only few HA reactive thymocytes are present in the F1 CD11c HA^{low} mice due to a strong negative selection. Those clones show a high incidence for developing further into Treg lineage. F1 CD11c HA^{high} mice show an even stronger negative selection. In this system only very few clones are not deleted

but could be selected into Treg lineage. However, it is well possible that these few Treg cells after leaving the thymus are overwhelmed by the inflammatory milieu in the periphery especially in the young F1 CD11c HA^{high} mice and therefore are not able to perform adequate suppressive function. Some of them might proliferate over time and eventually contribute to the minor increase in 6.5^{+} Treg's in adult F1 CD11c HA^{high} mice.

Moreover, like in the thymus, practically no 6.5^{+} CD4 T cells expressing Foxp3 were detectable in the spleen of young F1 HA^{high} mice whereas around 35% of these were Foxp3 positive in young F1 HA^{low} mice. The absence of HA specific Treg's in young F1 HA^{high} mice might well be the cause of the anemia and the myeloid hyperplasia since mice that cannot generate Treg's show a similar phenotype [48-50]. The findings that the cytokine production by CD4 cells, the anemia and the myeloid hyperplasia can to large extent be overcome by the transfer of HA specific Treg's into newborns, but not by polyclonal Treg's strongly supports this hypothesis. The myeloid hyperplasia observed in young F1 HA^{high} mice is likely to be due to the relative high number of GM-CSF producing CD4 T cells. Moreover it could well be envisaged that the observed anemia is indirectly caused by the high number of GM-CSF producing CD4 T cells in that this causes an increase of myelopoiesis at expense of erythropoiesis in the bone marrow.

In adult F1 HA^{high} mice the relative numbers of IFN-γ and GM-CSF CD4 cells decreased and a normalization of the hematocrit values was observed. Moreover, a small but significant increase in HA specific Treg's was observed. Nevertheless, all adult F1 HA^{high} mice developed arthritis and had high titers of IgG ANA. In other words from young to adult the disease observed changed from an acute to a more chronic type. We should stress that HA-specific regulatory T cells transfer did not

improve the disease in adult F1 HA^{high} mice. One explanation is the possible disappearance or exhaustion of transferred cells over time. However, even if HA-specific Treg cells were to be transferred throughout life of F1 HA^{high} mice, it is very likely that the ratio of thymus output of HA-specific effector cells versus transferred HA-specific Treg cells would still be imbalanced towards effector cells. Therefore, our model would argue that, autoimmune diseases that are sustained by a constant thymus output of self-specific cells are unlikely to be successfully treated by one or several transfers of Treg cells.

Autoimmune disease was remarkably different in young versus adult F1 HA^{high} animals correlating with differences in the level of T cell activation. The difference is likely to be explained by the number of HA specific Treg's. In young F1 HA^{high} mice they are practically absent whereas in adult mice their number is low but significantly increased. This increased number of HA specific Treg's is likely to be responsible for the change in the observed autoimmunity disorder in these mice. The increase of these Treg's in adult mice might well be due to an IL-2 driven proliferative expansion of the very low numbers generated in the young thymus.

As described before, HA under control of the CD11c promoter was found to be expressed not only by DC's but as well, e.g., at a low level by B cells. The strong B cell activation observed in the F1 HA^{high} mice might at least in part be due to cognate interaction between HA specific T cells and $CD11c^{+}$ HA expressing B cells. Moreover, although less efficient B cell activation seems also to be due to the cognate interaction of HA specific T cells and B cells, that picked up HA molecules and processed them for presentation. The strong B cell activation in the F1 HA^{high} mice results in the formation of high titers of IgG ANA. However, whether the strong B cell activation is also involved in the development of arthritis is still unclear. We are

currently generating a B cell deficient BALB/c mouse in order to address this question. However, unlike in several other model systems [51, 52] arthritis could not be transferred by serum from diseased mice (data not shown).

Models of autoimmune disease caused by concomitant transgenic expression of a TCR together with cognate antigen have already been reported by others [28, 53-55]. Interestingly, when mice expressing HA under SV40 [56], Igκ [57], phosphoglycerate kinase [58, 59] or Aire [42] promoter are crossed with TCR HA mice, offspring develop normal without any signs of autoimmunity. In contrast, HA-specific cells in these mice are enriched for $Foxp3^+$ CD4 cells and/or IL-10 secreting cells as a result of regulatory T cell development in the thymus or effector cell conversion in the periphery. However, a recent publication showed that mice expressing a transgenic TCR specific for HA crossed to a transgenic mouse expressing HA under control of the MHC class II promoter developed arthritis [54]. Somehow surprising, the development in this model is not dependent on T cells escaping negative selection by expressing two TCR's since arthritis development was also observed on a *Rag* deficient background. Whether these double transgenic mice when they are young suffer from anemia has not been documented. Altogether, the comparison of all these double transgenic models suggests that the nature of the cell type expressing a self-antigen critically influences T cell biology.

Our results demonstrate, that not only the identity of the antigen presenting cells, but also the dose of antigen expression, influences the establishment of tolerance versus autoimmunity. Overall, it is well established that dendritic cells play a crucial role in thymic negative selection of T cells expressing a TCR with high affinity for MHC peptide complexes. Our data presented here, argue that also the avidity between TCR and MHC peptide complexes might play a role in thymic

selection in that high avidity interaction results in a more stringent negative selection, whereas low avidity interaction seems to support Treg formation.

Materials and Methods

Mice

BALB/c, BALB/c *Rag-2* deficient, Igκ HA BALB/c [34] mice and BALB/c mice expressing a transgenic αβ TCR specific for peptide 111 – 119 of HA [33, 60] were bred under SPF conditions in our animal unit. For the generation of transgenic mice expressing HA under control of the CD11c promoter, the complete coding region of the HA gene of influenza virus A/PR8/34 was PCR amplified from the cDNA clone pGEM-4-HA (kindly provided by Dr. A. Caton, Wistar Institute, Philadelphia, PA)[61]. The HA PCR product was inserted as a blunt-end product into the EcoRI site of rabbit β-globin gene of the CD11c-promoter vector as previously described [36], (the CD11c-promoter vector was kindly provided by Dr. T. Brocker, University of Munich, Germany). The plasmid DNA was linearized by XhoI and NotI digestion and injected into fertilized oocytes from BALB/c mice. HA transgenic mice were selected by PCR analysis using mouse tail DNA as a template and the following HA-specific primers: 5'-TCCCTCAGCTCCTCATAGTC-3' and 5'-GAAAGGACTCTGGATTTCCATG-3'. Of the nine different founder mice, five expressed the HA transgene as determined by RT-PCR and by FACS analysis using a rat anti HA monoclonal antibody for surface staining of dendritic cells (kindly provided by Dr. A. Caton, Wistar Institute, Philadelphia, PA; used as described in [34]) for surface staining of spleen cells. All founder mice expressed the HA transgene in a CD11c specific manner. Two founders that surface-expressed the HA antigen either at a high (CD11c HA^{high}) or a low (CD11c HA^{low}) level were used in the following experiments.

All animal experiments were approved by the State veterinary authorities of Basel (Kantonales Veterinäramt, Basel-Stadt).

Southern Blot analyses of HA transgene copy numbers

Genomic DNA of thymus-derived cells was isolated from different founders by phenolic extraction. 5 μg of DNA was digested over night with PvuII and separated on a 1.8% agarose gel. The gel was blotted onto a nylon membrane (Roche Diagnostics GmbH, Mannheim) over night and UV crosslinked (0.12 J/cm^2). A DIG-labeled probe specific for HA with a size of 373 bp was PCR amplified using the following primer set: forward primer 5'-GACAGCCACAACGGAAAACT-3' and reverse primer 5'-GGTATGAGCCCTCCTTCTCC-3'. As PCR template the HA gene of influenza virus A/PR8/34 cloned blunt-ended into the LXP plasmid via the EcoRI restriction site. Probe labeling, hybridization and chemiluminescence detection were performed according to standard protocols (Roche Diagnostics GmbH).

HA transgene expression in different cell lineages

cDC's, pDC's were derived from 7 day BM cultures (1x10^6 cells/ml, 200 ng/ml FLT3L) and were sorted according to their expression level of CD11c, MHCII and Siglec H [62]. NK cells were derived from 5 day BM cultures (1x10^6 cells/ml, 10^4 U/ml of recombinant mouse IL-2) [63]. B and T cells were sorted from spleen suspension *ex vivo* according to the expression of CD19 or CD3. RNA was prepared from all cell samples using Tri Reagent (MRC). 400 ng RNA were used in a first strand synthesis reaction. Expression of the HA transgene was revealed by qPCR using the forward primer 5'-GACAGCCACAACGGAAAACT-3' and the reverse primer 5'-CCCTCAGCTCCTCATAGTCG-3' (amplicon: 205 bp). HPRT was amplified as endogenous control gene and results were shown as fold expression referring to cDC cDNA derived from CD11c HA^{high} mice.

Antibodies and flow cytometric analysis

FITC-, PE-, APC- or biotin-conjugated mAb specific for CD4 (L3T4), CD8α (53-6.7), CD69 (H1.2F3), CD44 (IM7), CD62L (MEL-14), anti-TCR β-chain (H57-597), CD19 (6D5), Gr1 (RB6-8C5), CD11b (M1/70), Vβ8 (F23.1) and Vα2 (B20.1) were purchased from BD Bioscience (Allschwil, Switzerland). Streptavidin, conjugated to PE, APC or PE/Cy7 were also purchased from BD Bioscience. Anti-FoxP3 (FJK-16s), anti-IFN-γ (XMG1.2) and anti-GM-CSF (MPI-22E9) were purchased from eBioscience. The rat anti-CD19 (ID3) monoclonal antibody and the rat anti-TCR HA clonotype specific mAb 6.5 [33] were purified from hybridoma supernatants and labeled with biotin in our laboratory by standard methods. Flow cytometry was performed using a ™FACS Calibur (BD Biosience) and sorting using the ™FACS-ARIA (BD Biosience). Data were analyzed using the ™Cell Quest Pro Software (BD Bioscience). CFSE labeling was performed according to standard procedures; briefly, sorted cells were labeled in the dark at 37°C for 5 minutes. In Figure 1 and 2, dendritic cells were enriched from thymus or spleen as described elsewhere [62].

Immunohistochemical and histological analyses

Spleens were snap frozen and embedded in OCT-compound (Sakura, Zoetermeer, NL), and 5 μm sections were prepared. Sections were fixed in acetone for 10 min and then air dried for 60 min. To analyze B and T cell organization in spleen and to determine the presence of germinal centers in spleen, sections were incubated with biotinylated anti-IgM (clone M41) and FITC labeled anti-Thy1 (T24) or with anti-IgM FITC (M41) and biotinylated peanut agglutinin (PNA) (Vector, Burlingame, CA) for 30 min. After 20 min washing in PBS, anti-IgM and PNA binding were revealed with PE conjugated streptavidin (SouthernBiotech, Birmingham, AL). Paw joints were detached and fixed in 4% buffered formalin, decalcified in 20% EDTA and

embedded in paraffin. Standard frontal sections of the joints were stained with hematoxylin and eosin (H&E) according to standard procedures.

Hematocrit determination

Mice were bled into Na-heparinized micro-haematocrit tubes (Huber&Co.AG, Reinach). Red blood cells were separated from plasma by a 10 min centrifugation step at 10,000 rpm in a Haemofuge A (Heraeus AG, Switzerland) and hematocrit values were calculated as percentage of red blood cell volume of whole blood sample volume.

IgG anti-nuclear autoantibody determination

IgG autoantibodies against nuclear antigens were detected by an indirect immunofluorescence technique (described in [64]), using a FITC labeled goat anti-mouse IgG (Jackson Immunoresearch, Milan Analytica, La Roche, CH); 5 μm cryosections of kidneys from *Rag-2* deficient mice were used as substrate. The titer was defined as the highest serum dilution still giving a positive nuclear staining.

PMA/ionomycine stimulation

Total lymphocytes were stimulated at 37°C during 4 hours using 1 μg/ml ionomycine (Sigma-Aldrich) and 5ng/ml phorbol-12-myristate-13-acetate (PMA) in the presence of 10 μg/ml brefeldin A (Calbiochem). Cells were harvested and stained by standard intracellular staining procedure. In brief, surface staining was followed by a fixation step using 2% paraformaldehyde in PBS followed by intracellular staining in FACS buffer containing 0.5% saponin.

T cell proliferation assays

T cells expressing or not expressing the transgenic HA specific TCR recognized by the anti- clonotypic 6.5 mAb were sorted from TCR HA mice. Various numbers of these cells were co-cultured with 5 x 10^5 2400 rad irradiated spleen cells from CD11c

HA^{high} mice or WT BALB/c mice. At day 3 of culture proliferation was determined by ^{3}[H]-Thymidine uptake. To test the ability of $CD11c^{+}$ B cells to stimulate HA specific T cells, $CD11c^{+}$ $CD19^{+}$, $CD11c^{bright}$ $CD19^{-}$ and $CD11c^{-}$ $CD19^{+}$ cells were sorted from single transgenic CD11c HA^{high}, CD11c HA^{low} and WT BALB/c mice and various numbers of these were co-cultured with 2 x 10^{5} lymph node cells from TCR HA mice. At day 4 of culture proliferation was determined by ^{3}[H]-Thymidine uptake.

Treg transfer into newborn F1 HA^{high} mice

Treg cells were purified from (Igκ HA x TCR HA)F1 mice by magnetic enrichment of CD4 cells followed by cell sorting of $CD25^{+}$, 6.5^{+} CD4 T cells using the FACS ARIA. As control, Treg cells were isolated from WT BALB/c mice. 1.5 x 10^{5} HA specific or polyclonal Treg's were injected i.p. into newborn F1 HA^{high} mice.

BM chimaera

Bone marrow (BM) was isolated from femur and tibia of TCR HA donor mice. BM cell suspension was incubated with anti-CD4 (RL172) and anti-CD8 mAb (31M) prior to complement depletion of T cells by incubating the sample with rabbit serum (1:20 in DMEM) for 30 min at 37°C. The procedure was followed by erythrocyte lysis buffer treatment. Single transgenic BALB/c, CD11c HA^{high}, CD11c HA^{low} and Igκ HA recipients were lethally irradiated (750 rad) prior to injection of $4*10^{6}$ T cell depleted BM cells/mouse. BM chimaeras were analyzed in week 6 after BM reconstitution.

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Conflict of interest.

The authors have no financial conflicts of interest.

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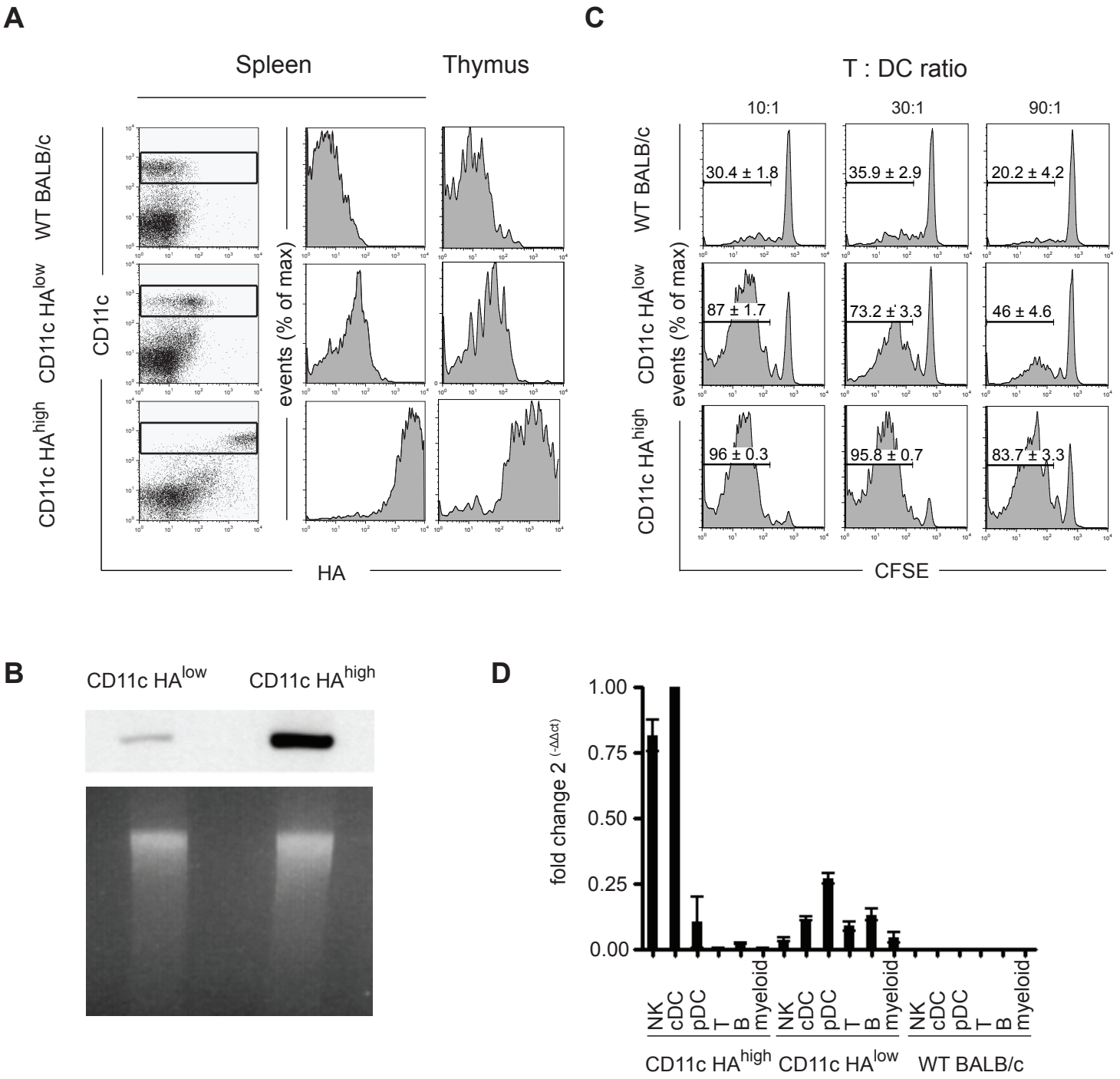


Figure 1

Expression of HA by CD11c positive cells in the spleen and thymus of CD11c HA transgenic mice. (A) Dot plots show CD11c versus HA expression on the dendritic cells enriched fractions from the spleen of WT BALB/c, CD11c HA^{low} and CD11c HA^{high} transgenic mice. Histograms show HA expression on CD11c high cells from the spleen (left) or thymus (right) of WT BALB/c, CD11c HA^{low} and CD11c HA^{high} transgenic mice. (B) The difference in copy number of the inserted HA transgene in CD11c HA^{low} and CD11c HA^{high} founders was assessed by Southern blotting using a DIG-labeled HA specific probe (373 bp). Below is a picture of the Red Safe® stained gel prior to transfer to the nylon membrane as loading control. (C) Sorted- CFSE-labeled 6.5^{+} CD4^{+} cells from TCR HA mice were co-cultured with sorted and irradiated DCs from WT BALB/c, CD11c HA^{low} or CD11c HA^{high} mice in several T cell to dendritic cell ratios (T : DC). T cell proliferation was measured by monitoring CFSE-dilution at day 4. (D) The expression of HA antigen under the CD11c promoter was analyzed by qPCR using cDNA of sorted NK, cDC, pDC, T, B and myeloid cell populations of the CD11cHA^{high} (to the left), CD11cHA^{low} (middle) and WT BALB/c (to the right) mouse strains. For each sample HA and HPRT (control) cDNA was PCR amplified and the fold change $2^{(-\Delta\Delta ct)}$ was determined referring to the cDC sample derived from CD11cHA^{high} mice. All panels show representative experiments, which have been performed at least 3 times on separate and independent occasions.

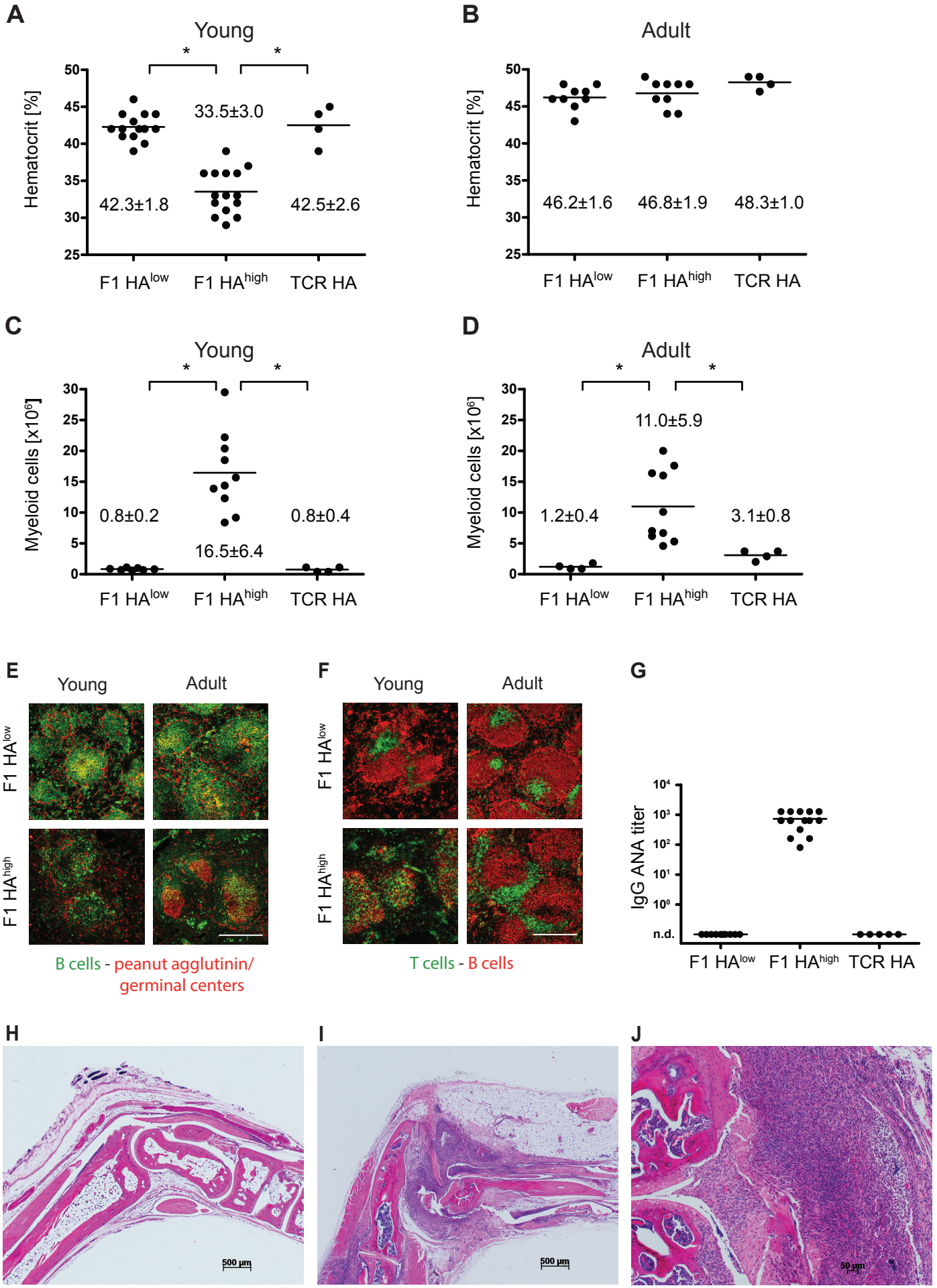

A
Young
Hematocrit [%]
50
45
40
35
30
25
*
*
33.5±3.0
42.3±1.8
42.5±2.6
F1 HA^low
F1 HA^high
TCR HA
B
Adult
Hematocrit [%]
46.2±1.6
46.8±1.9
48.3±1.0
F1 HA^low
F1 HA^high
TCR HA
C
Young
Myeloid cells [x10^6]
30
25
20
15
10
5
0
*
*
0.8±0.2
16.5±6.4
0.8±0.4
F1 HA^low
F1 HA^high
TCR HA
D
Adult
Myeloid cells [x10^6]
*
*
11.0±5.9
1.2±0.4
3.1±0.8
F1 HA^low
F1 HA^high
TCR HA
E
Young
Adult
F1 HA^low
F1 HA^high
B cells - peanut agglutinin/
germinal centers
F
Young
Adult
F1 HA^low
F1 HA^high
T cells - B cells
G
IgG ANA titer
10^4
10^3
10^2
10^1
10^0
n.d.
F1 HA^low
F1 HA^high
TCR HA
H
500 µm
I
500 µm
J
50 µm

Figure 2

Disease indicators in the serum and tissues of young (week 4) and adult (> week 8) double transgenic (TCR HAxCD11c HA) F1 mice (A-D) and immunoflourescence of spleen sections, serum anti-nuclear antibodies and histology of joints from double transgenic mice (E-J). (A-B) Hematocrit values for young (A) and adult (B) mice of double transgenic F1 HA^{low}, F1 HA^{high} and single transgenic TCR HA mice. Hematocrit values of healthy WT mice are within the range of 40-50%. (C-D) Total number of myeloid cells ($Gr1^+$, $CD11b^+$) in the spleen of young (C) and adult (D) animals of the same mouse strains. Asterisks indicate $P< 0.01$ for the groups compared as indicated by the horizontal bars. (E) Spleen sections from young and adult double transgenic F1 HA^{low}, F1 HA^{high} mice stained for IgM (green; monoclonal anti-μ) and peanut agglutinin (red) to reveal B cell organization and germinal center formation (scale bar: 350 μm). (F) Spleen sections from young and adult double transgenic F1 HA^{low}, F1 HA^{high} stained for IgM (red; monoclonal anti-μ) and T cells (green; monoclonal anti-Thy1) to examine B and T cell organization (scale bar: 350 μm). (G) Titers of IgG anti-nuclear autoantibodies (ANA) in the serum of adult double transgenic F1 HA^{low}, F1 HA^{high} and single transgenic TCR HA mice. Each symbol represents an individual mouse. The titer is defined as the highest serum dilution still giving a positive nuclear staining in the indirect-immunofluorescence assay (nd: not detectable level). (H) Histological section of normal joint with no inflammation (x25 H&E). (I) Joint from an adult F1 HA^{high} mouse with severe arthritis (x25 H&E), showing (J) granulation tissue, synovial destruction as well as mononuclear inflammation (x100 H&E). All panels show representative experiments, which have been performed at least 3 times on separate and independent occasions.

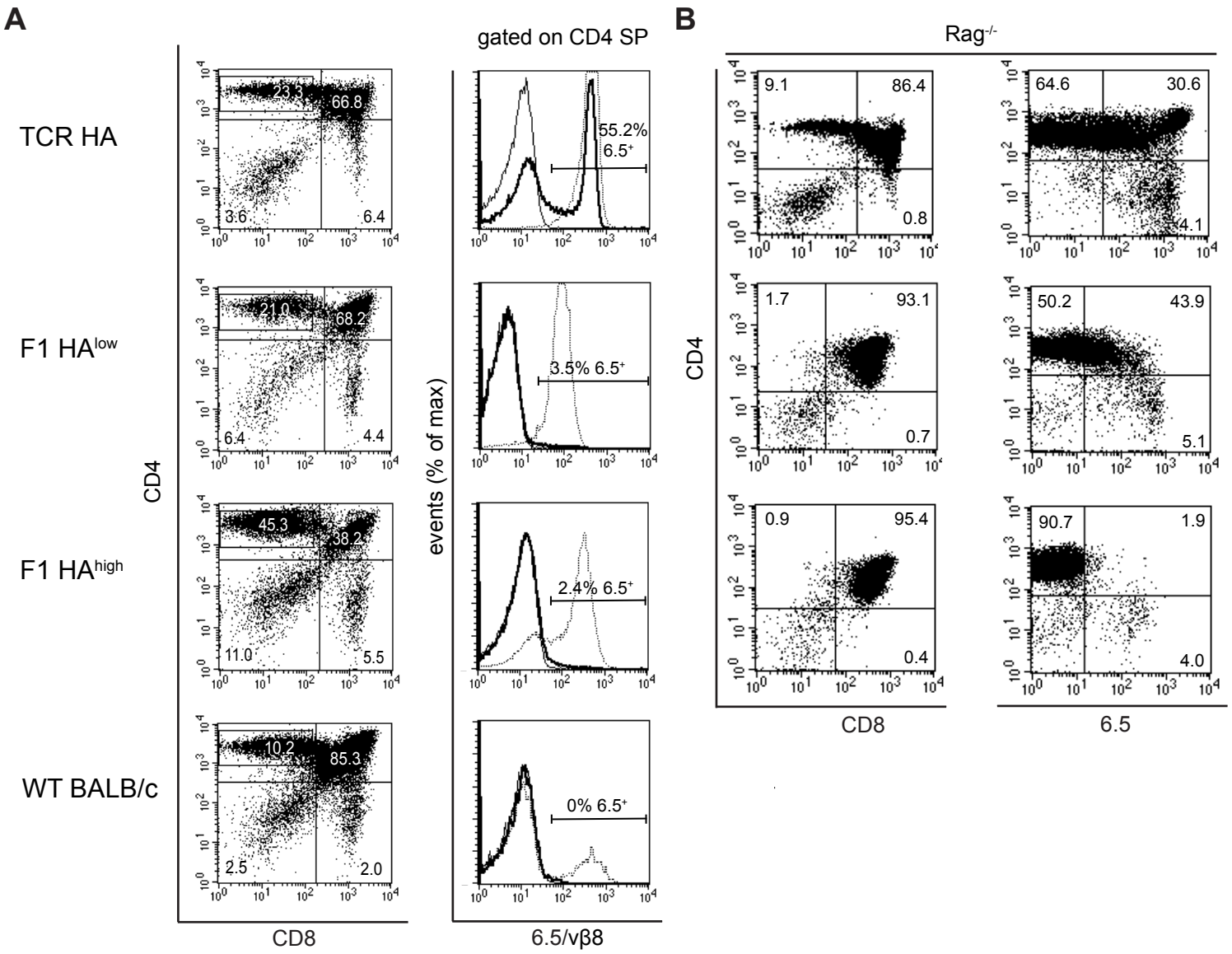


Figure 3

Negative selection of HA-specific thymocytes in the thymi of F1 HA^{low} and F1 HA^{high} double transgenic mice. (A) FACS analyses of the expression of CD4 and CD8 on thymocytes from single transgenic TCR HA, double transgenic F1 HA^{low}, F1 HA^{high} and WT BALB/c mice (to the left). Histograms (to the right) showing the expression of the transgenic HA specific TCR (vβ8 and vα4, detected by the idiotype specific antibody 6.5, bold line) or transgenic vβ8 chain only (dashed line) versus unstained cells (solid line). Histograms were generated after gating on CD4sp thymocytes as indicated in the dot plots to the left. (B) FACS analyses of the expression of CD4 and CD8 on thymocytes from single transgenic TCR HA, double transgenic F1 HA^{low} and F1 HA^{high} mice on a Rag$^{-/-}$ genetic background (to the left). FACS analyses of the expression of CD4 and HA-specific TCR (6.5) on thymocytes from single transgenic TCR HA, double transgenic F1 HA^{low} and F1 HA^{high} mice on a Rag$^{-/-}$ genetic background (to the right). All panels show representative experiments, which have been performed at least 3 times on separate and independent occasions.

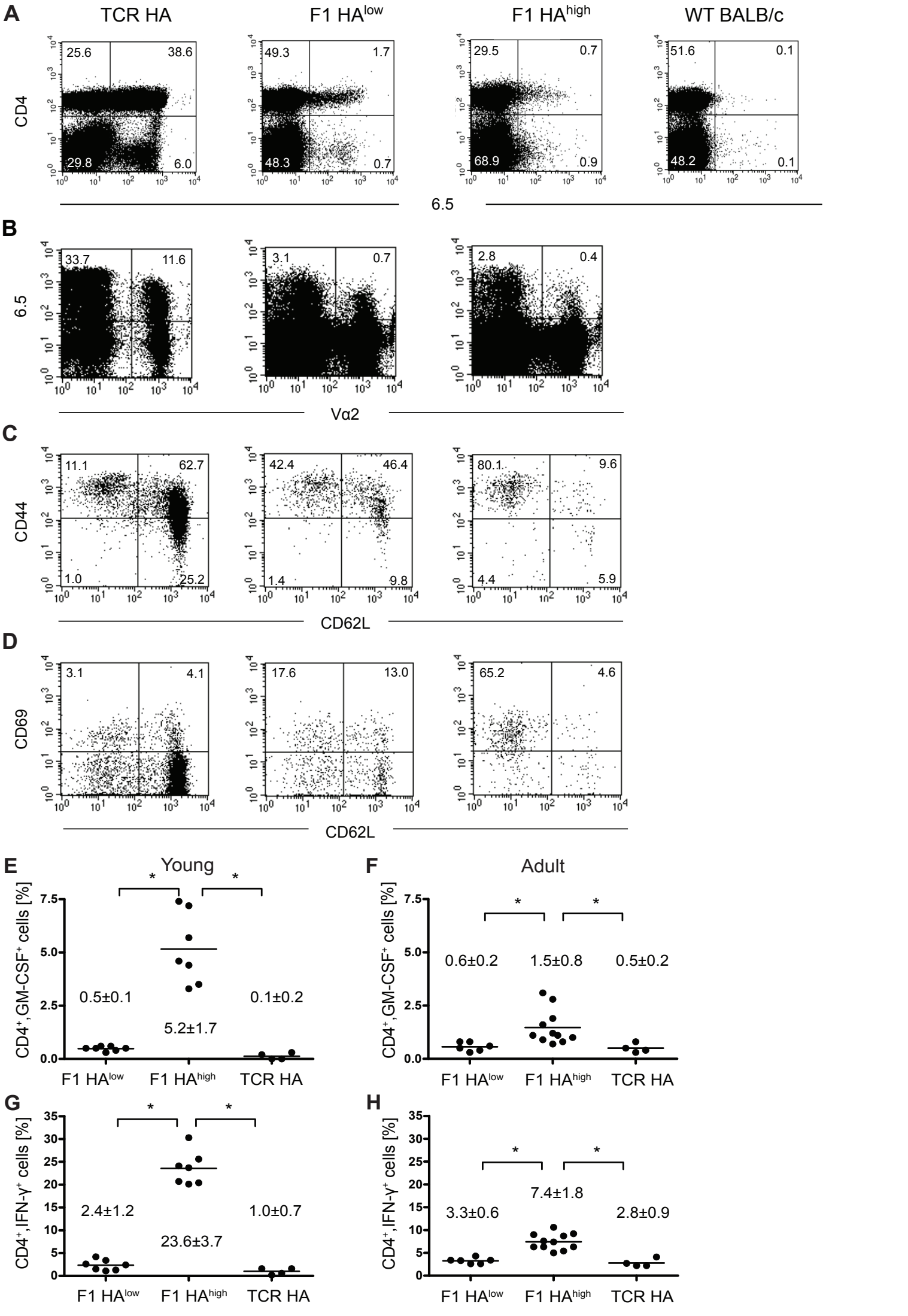

A
TCR HA
F1 HA^low
F1 HA^high
WT BALB/c
CD4
25.6
38.6
29.8
6.0
49.3
1.7
48.3
0.7
29.5
0.7
68.9
0.9
51.6
0.1
48.2
0.1
6.5
B
6.5
33.7
11.6
3.1
0.7
2.8
0.4
Vα2
C
CD44
11.1
62.7
1.0
25.2
42.4
46.4
1.4
9.8
80.1
9.6
4.4
5.9
CD62L
D
CD69
3.1
4.1
17.6
13.0
65.2
4.6
CD62L
E
Young
CD4+,GM-CSF+ cells [%]
0.5±0.1
5.2±1.7
0.1±0.2
F1 HA^low
F1 HA^high
TCR HA
F
Adult
0.6±0.2
1.5±0.8
0.5±0.2
G
CD4+,IFN-γ+ cells [%]
2.4±1.2
23.6±3.7
1.0±0.7
H
3.3±0.6
7.4±1.8
2.8±0.9

Figure 4

Absence of peripheral tolerance and activation of HA-specific CD4 T cells in F1 HA^{high} mice. (A) Dot plots show CD4 versus HA-specific TCR (6.5) expression on cells from lymph nodes of adult single transgenic TCR HA (left panel), adult double transgenic F1 HA^{low} (middle, left panel), F1 HA^{high} (middle, right panel) and adult WT BALB/c (right panel) mice. (B) Endogenous Vα2 and HA-specific TCR (6.5) expression by CD4 positive cells from lymph nodes of single transgenic TCR HA, double transgenic F1 HA^{low} and F1 HA^{high} mice. (C-D) For further analysis cells were gated on $CD4^{+}6.5^{+}$ cells and examined for naïve ($CD62L^{+}$ and $CD44^{intermediate/low}$) or activated/memory ($CD62L^{-}$ and $CD44^{high}$) cell phenotype (C) and for expression of the early activation marker CD69 (D). (E-H) Inflammatory cytokine GM-CSF (E- F) and IFN-γ (G-H) secretion after PMA/ionomycine stimulation of $CD4^{+}$ spleen cells from 4 week (E- G) or 8 week (F-H) old mice. Asterisks indicate $P< 0.02$ for the groups compared as indicated by the horizontal bars. All panels show representative experiments, which have been performed at least 3 times on separate and independent occasions.

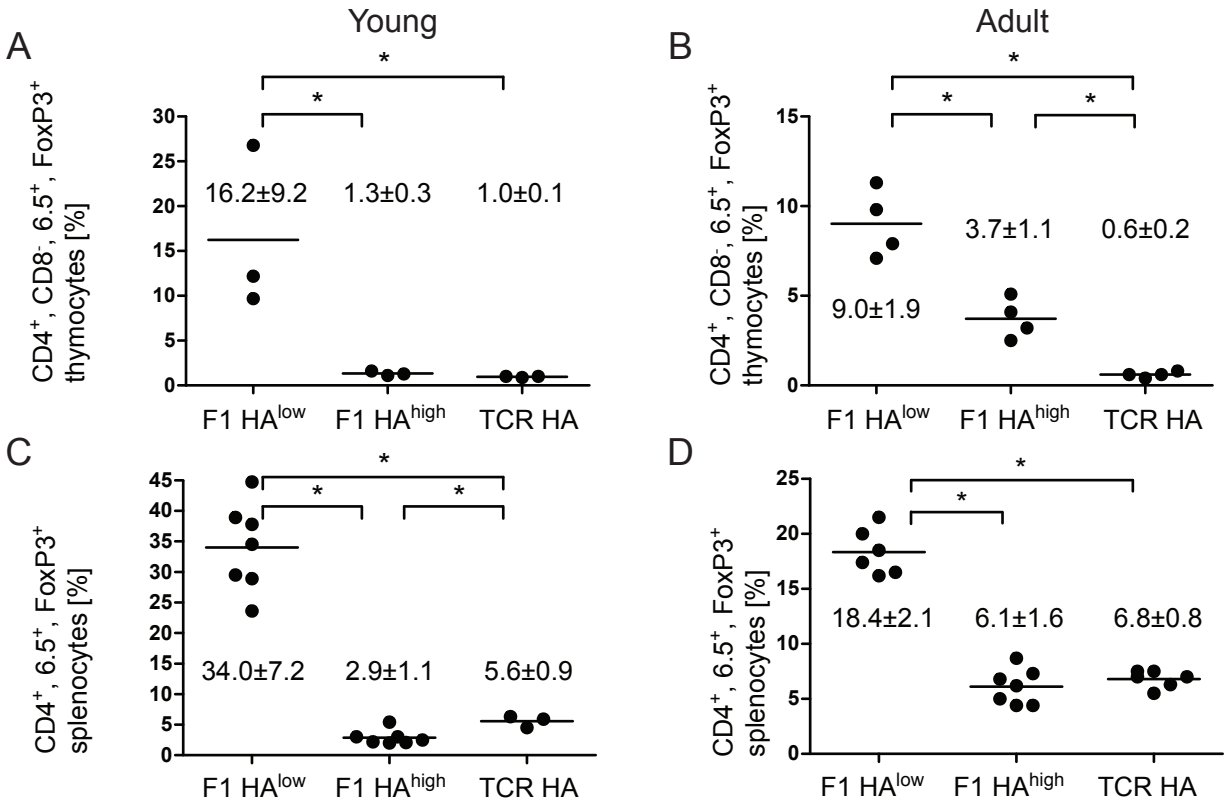


Figure 5

Autoimmunity correlates with poor HA-specific Treg development in F1 HA^{high} mice. (A-B) Histograms show the percentages of FoxP3 positive cells among HA-specific (6.5 positive) CD4sp cells from the thymus of young (A) or adult (B) F1 HA^{low}, F1 HA^{high} or TCR HA transgenic mice. (C-D) Histograms show the percentages of FoxP3 positive cells among HA-specific (6.5 positive) CD4 cells from the spleen of young (C) or adult (D) F1 HA^{low}, F1 HA^{high} or TCR HA transgenic mice. Dots represent individual animals and the mean ± SD is given in the plots for the different groups of mice. Asterisks indicate $P < 0.05$ for the groups compared as indicated by the horizontal bars. All panels show representative experiments, which have been performed at least 3 times on separate and independent occasions.

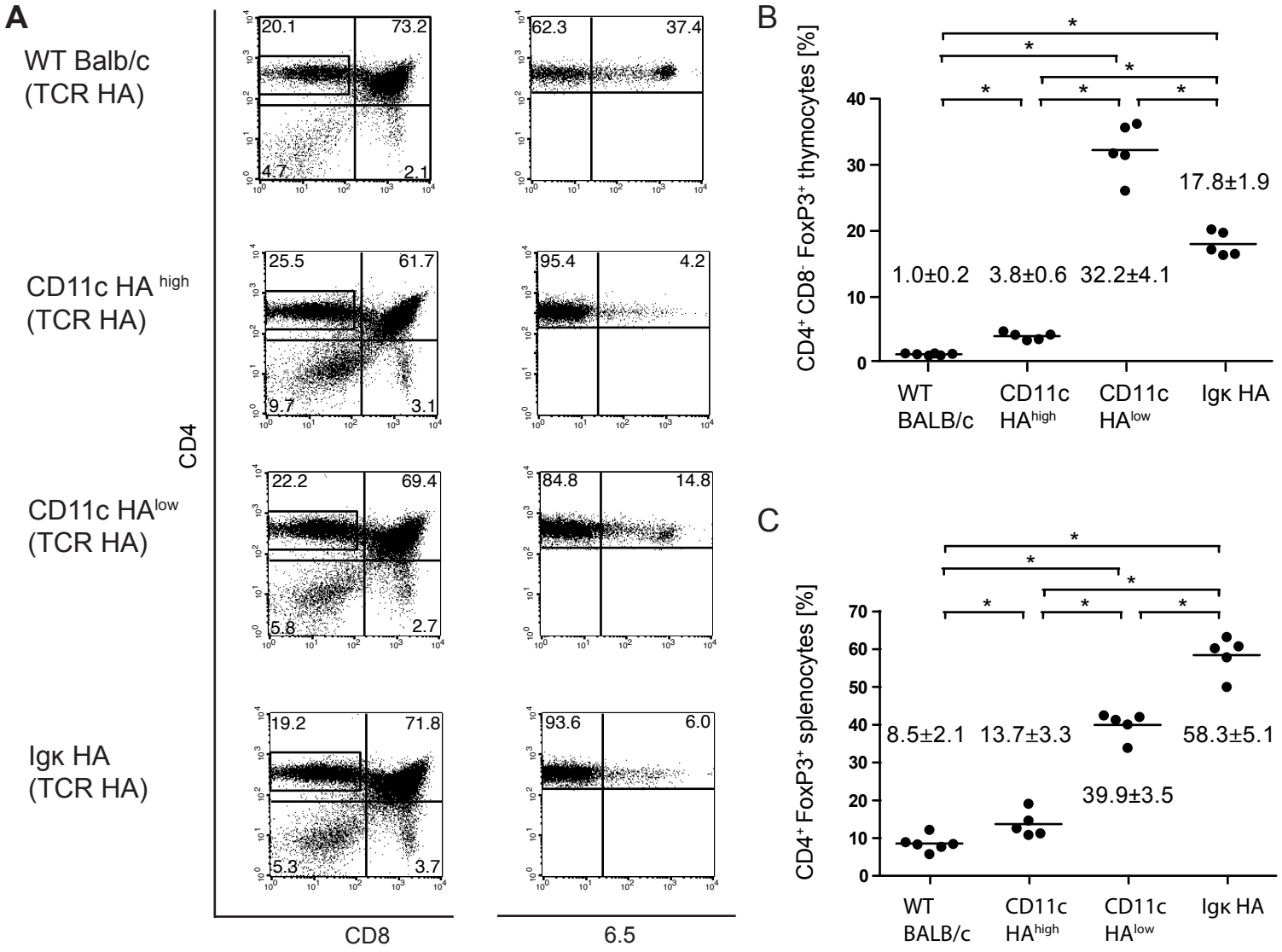


Figure 6

TECs express the HA transgene and contribute to thymic selection. (A) FACS analyses of the expression of CD4 and CD8 on thymocytes from WT BALB/c, single transgenic CD11c HA^{low}, CD11c HA^{high} and Igκ HA lethally irradiated hosts reconstituted with BM derived from TCR HA mice (to the left). To the right, FACS analyses of the expression of CD4 and HA-specific TCR (6.5) on CD4sp thymocytes of respective chimaeras. (B-C) Histograms show percentages of FoxP3 positive cells among HA-specific (6.5 positive) CD4sp cells from the thymus (B) or spleen (C) of WT BALB/c, CD11c HA^{high}, CD11c HA^{low} or Igκ HA chimaeras reconstituted with BM derived from TCR HA mice. Dots represent individual animals and the mean ± SD is given in the plots for the different groups of mice. Asterisks indicate $P<0.05$ for the groups compared as indicated by the horizontal bars. All panels show representative experiments, which have been performed at least 3 times on separate and independent occasions.

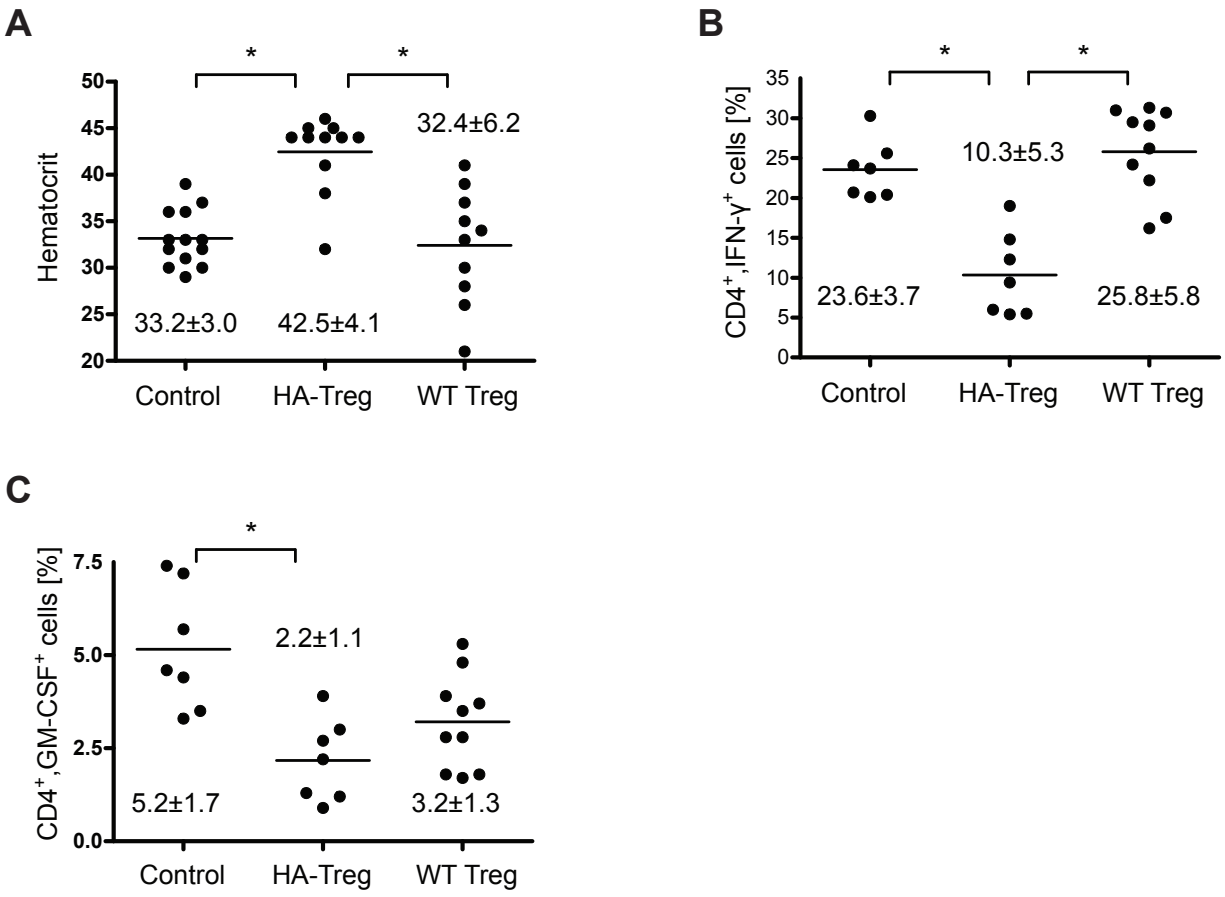
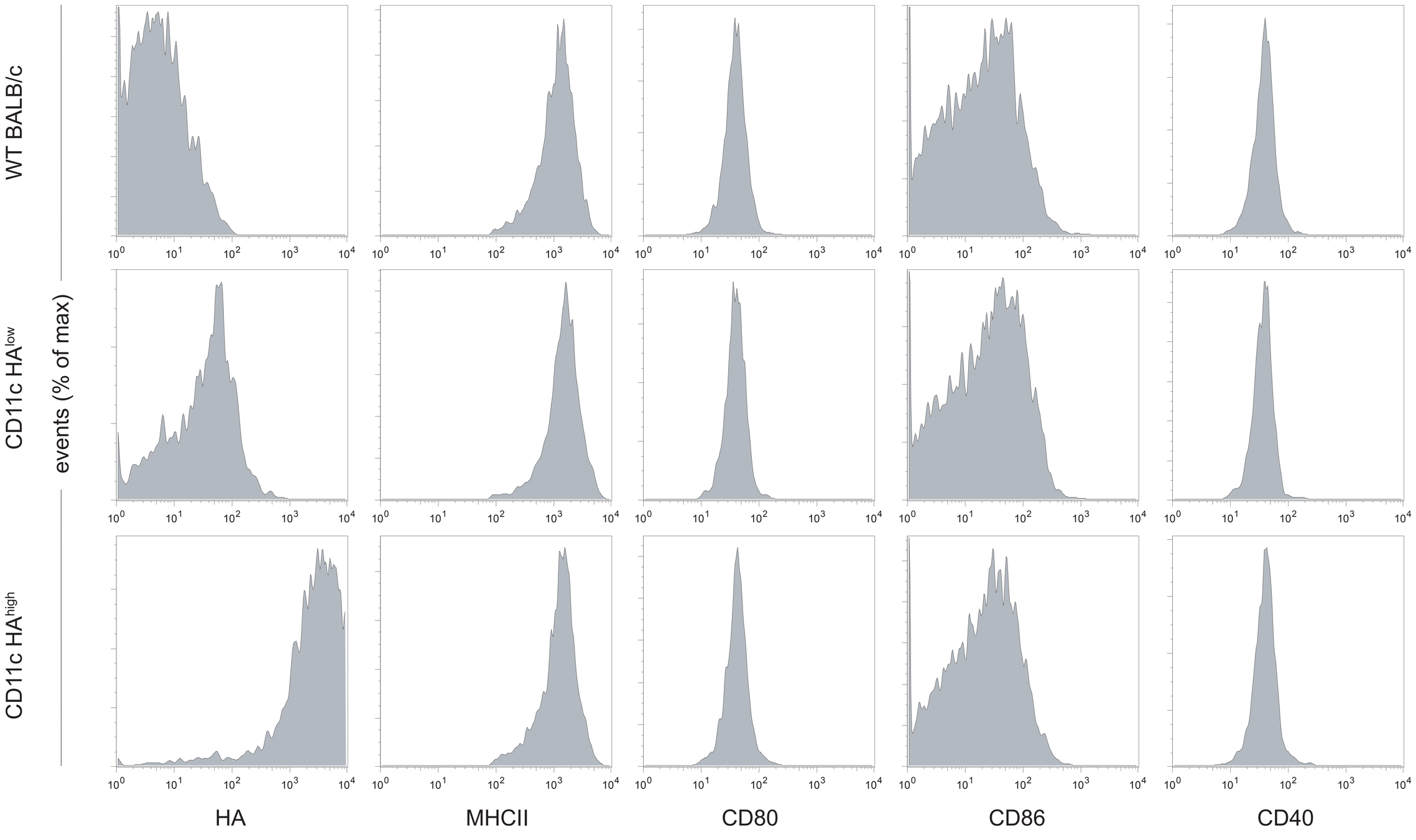


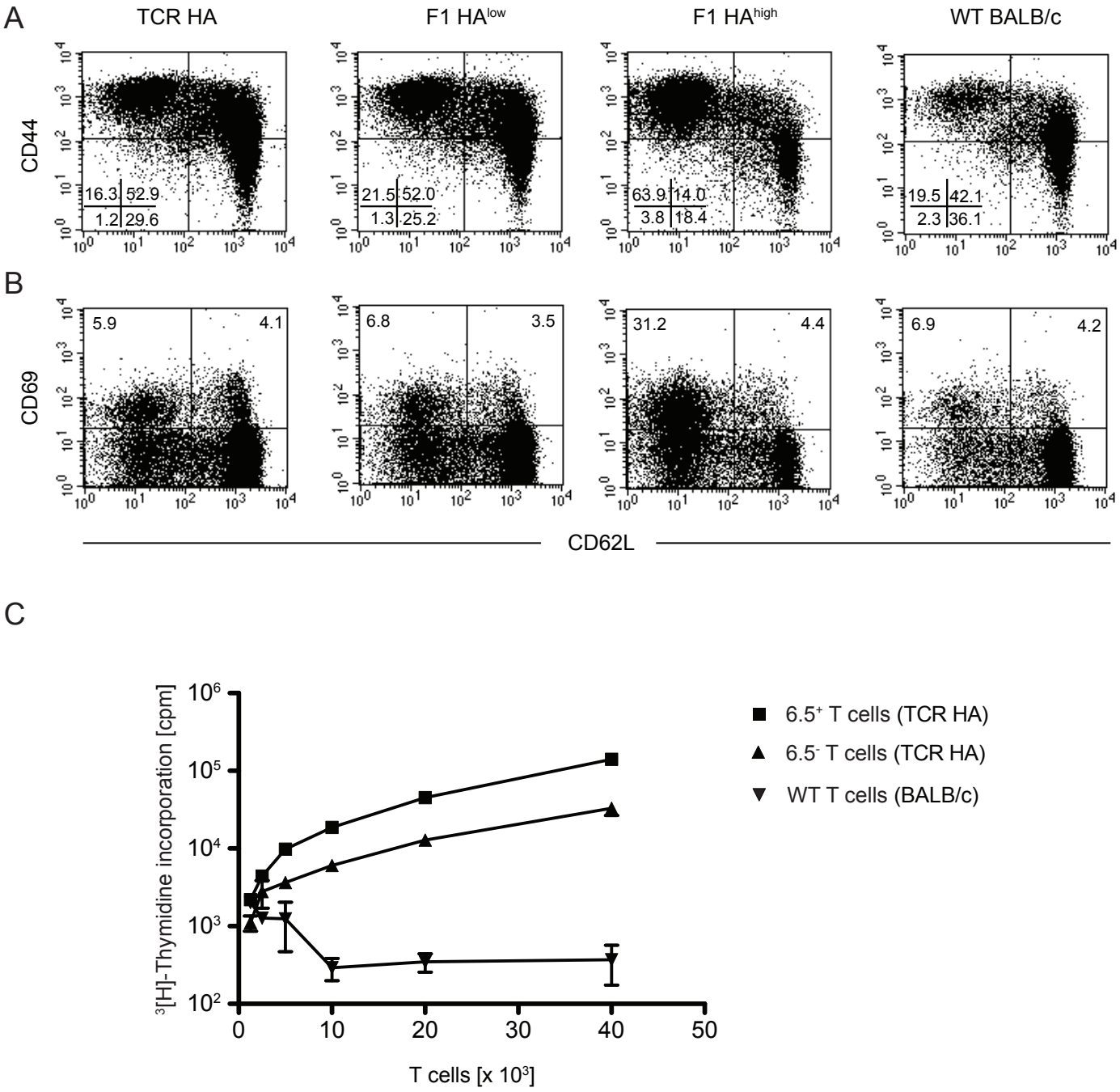
Figure 7

Transfer of HA specific Tregs into newborn F1 HA^{high} mice. Newborn double transgenic F1 HA^{high} mice were injected i.p. with sorted CD4^{+}CD25^{high} Treg cells from (Igκ HAxTCR HA)F1 mice (HA Treg) or from WT BALB/c mice (WT Treg). Untreated F1 HA^{high} mice were used as controls. After 4 weeks hematocrit values (A) as well as the fraction of CD4^{+} spleen cells secreting IFN-γ (B) or GM-CSF (C) upon PMA/ionomycine stimulation were determined. Dots represent individual animals and the mean ± SD is given below for the different groups of mice. Asterisks indicate $P < 0.01$ for the groups compared as indicated by the horizontal bars. All panels show representative experiments, which have been performed at least 3 times on separate and independent occasions.



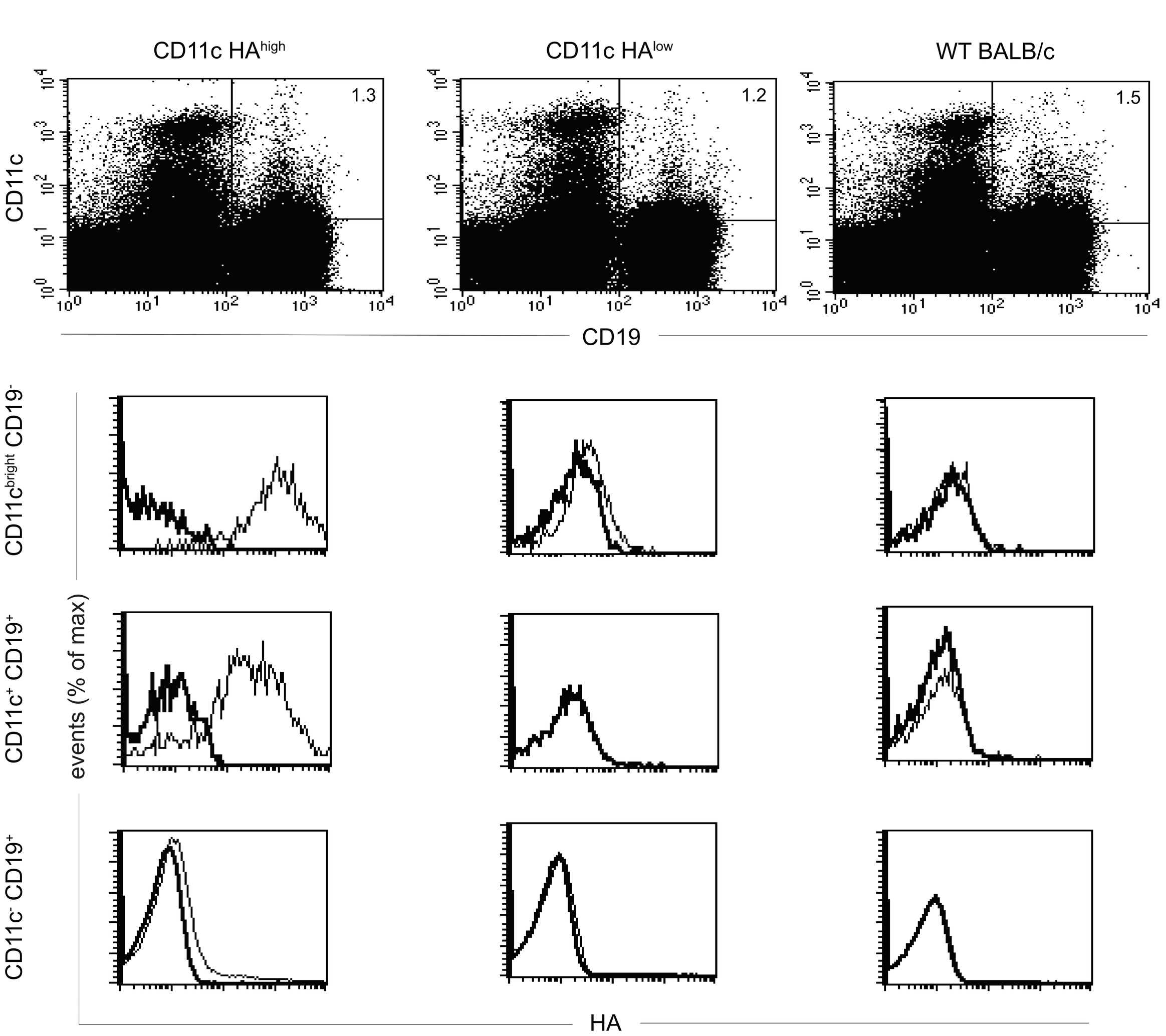
Supplemental Figure 1

No phenotypic changes of dendritic cells from CD11c HA transgenic mice. Histograms show surface expression of HA, MHC II, CD80, CD86 and CD40 on isolated CD11c$^+$ spleen cells from WT BALB/c, CD11c HAlow and CD11c HAhigh transgenic mice. All panels show representative experiments, which have been performed at least 3 times on separate and independent occasions.



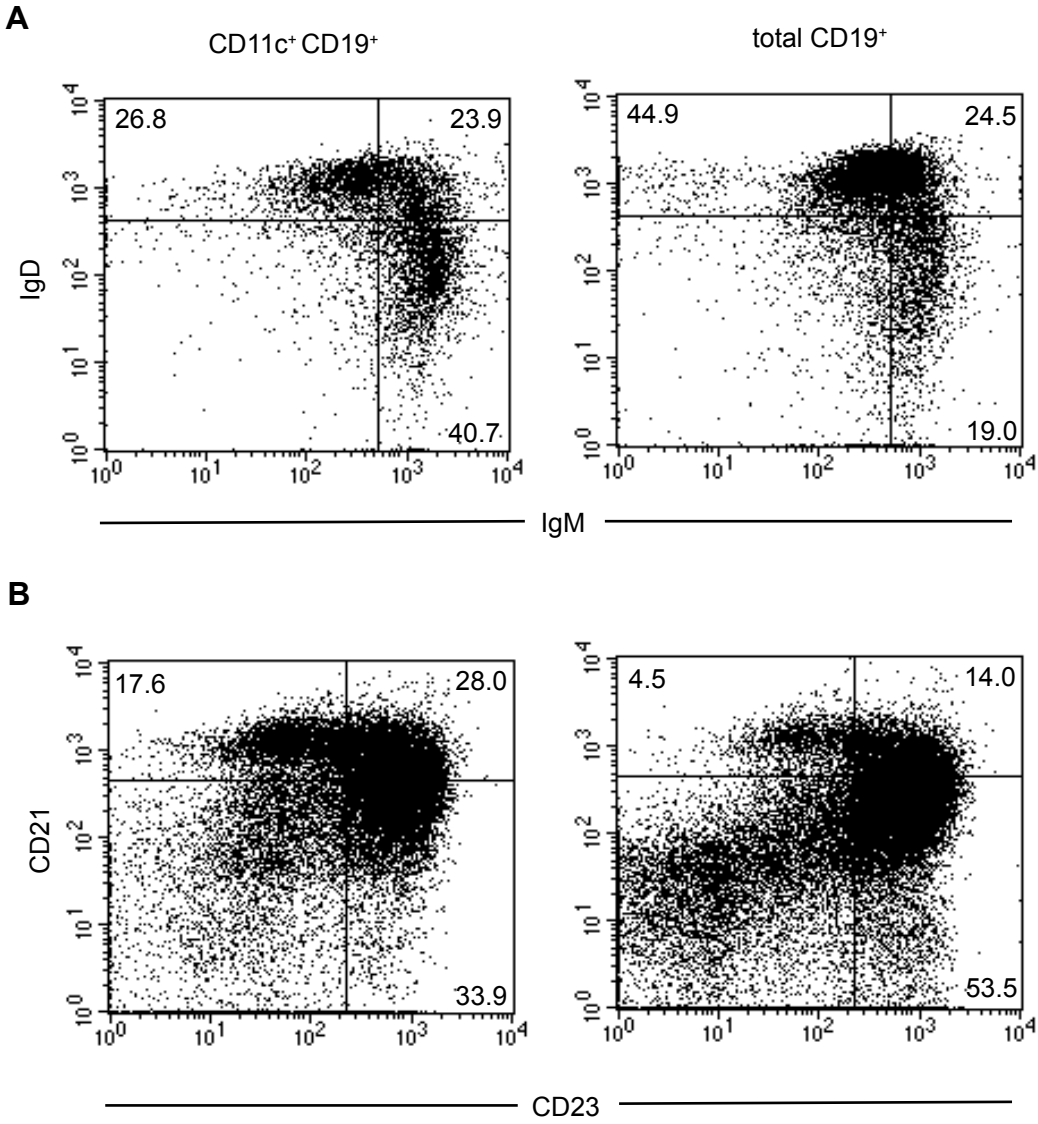
Supplemental Figure 2

Activation of CD4 T cell population in the periphery of F1 HA^{high} transgenic mice. (A) Dot plots show surface expression of CD62L and CD44 or (B) CD62L and CD69 on CD4$^+$ lymph node cells from adult single transgenic TCR HA mice, adult double transgenic F1 HA^{low}, F1 HA^{high} mice and adult WT BALB/c mice. (C) Sorted CD4$^+$6.5$^+$ and CD4$^+$6.5$^-$ from a single transgenic TCR HA mouse or sorted CD4$^+$ T cells from a WT BALB/c mouse were cultured for 3 days with irradiated spleen cells from a single transgenic CD11c HA^{high} mouse as a source of APC. Proliferation was determined by ^{3}[H]-Thymidine uptake. All panels show representative experiments, which have been performed at least 3 times on separate and independent occasions.



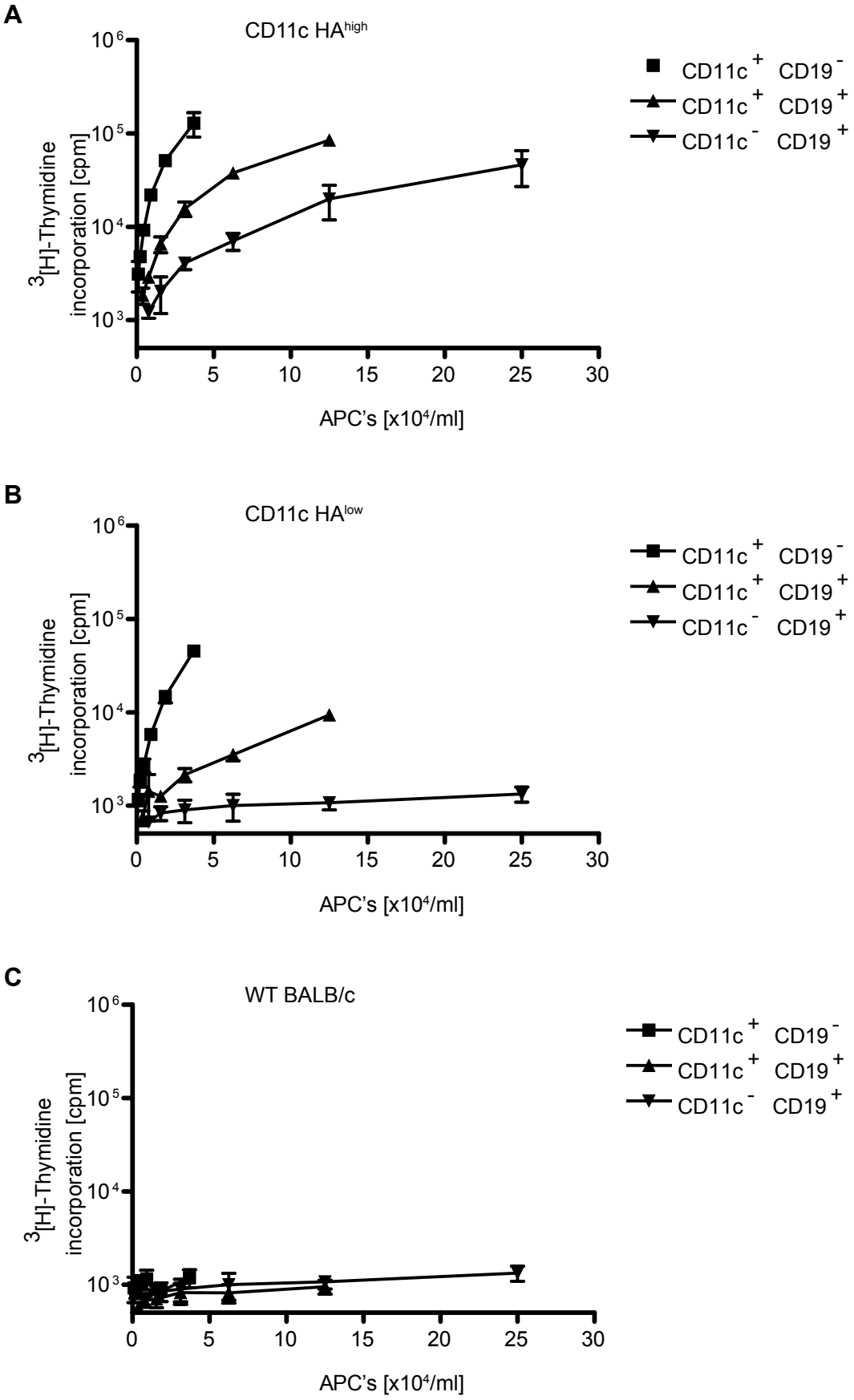
Supplemental Figure 3

HA expression on dendritic cells and B cells from CD11c HA^{high} transgenic mice versus CD11c HA^{low} transgenic and WT mice. (A) CD11c and CD19 expression profiles of CD11c HA^{high} (left panel), CD11c HA^{low} (middle panel) and WT BALB/c (right panel) mice were examined and (B) three distinguishable antigen presenting cell populations were analyzed for HA expression: DCs (CD11c^{bright}CD19^{-}; upper histograms), CD11c^{+} B cells (CD11c^{+}CD19^{+}; histograms in the middle) and B cells (CD11c^{-}CD19^{+}; lower histograms) (solid line: cells unstained for HA; faint line: HA-stained cells). All panels show representative experiments, which have been performed at least 3 times on separate and independent occasions.



Supplemental Figure 4

Enrichment of marginal zone B cells in the CD11c^{+}CD19^{+} B cell population in CD11c HA^{high} mice. (A-B) CD11c^{+} B cells (CD11c^{+}CD19^{+}; to the left) isolated from CD11c HA^{high} mice have an increased ratio for marginal zone B cells (CD21^{high}, CD23^{low}, IgM^{high}, IgD^{low}) to follicular B cells (CD21^{+}, CD23^{+}, IgM^{low}, IgD^{high}) compared to the total CD19^{+} cell population (to the right). All panels show representative experiments, which have been performed at least 3 times on separate and independent occasions.



Supplemental Figure 5

CD11c expressing B cells stimulate HA specific T cells. Sorted DC's ($CD11c^+CD19^-$), $CD11c^+$ B cells ($CD11c^+CD19^+$) and B cells ($CD11c^-CD19^+$) from the spleen of CD11c HA^{high} (A), CD11c HA^{low} (B) or WT BALB/c (C) mice were used for in vitro stimulation of HA-specific T cells (total lymph node cells isolated from TCR HA mice). The extent of proliferation after 3 days was determined by ^{3}[H]-Thymidine incorporation. All panels show representative experiments, which have been performed at least 3 times on separate and independent occasions.

IV

The development of autoimmune features in aging mice is closely associated with alterations of the peripheral CD4 T cell compartment

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Abbreviations

ANA, anti-nuclear antibody

SLE, Systemic lupus erythematosus

Tx, thymectomy

PNA, Peanut agglutinin

Summary

Some signs of potential autoimmunity, such as the presence of anti-nuclear antibodies (ANA) or rheumatoid factor (RF) become more prevalent with age. In most cases, these elderly people remain healthy. In the study reported here we have investigated whether the same holds true for inbred strains of mice. We found, that when they reach the age of 12 months, almost all mice of the C57BL/6 (B6) strain spontaneously produce high titers of IgG ANA. At this time, large numbers of germinal centers are present in the spleen, IgG deposition can be found in kidney glomeruli and lymphocyte infiltrates are found in the salivary glands. Despite all these signs of a potential autoimmune response, the mice remain healthy.

In contrast to B6 mice, DBA/2 mice do not produce IgG ANA's at that age. The F_1 hybrids of these two strains (BDF_1), show an intermediate incidence and lower titers of IgG ANA, pointing out the importance of genetic background and suggesting a mechanism of suppression of ANA production in DBA/2 mice. The production of ANA is CD4 T cell dependent, since B6 mice deficient for MHC class II do not produce IgG ANA's upon ageing. Experiments with irradiated bone marrow chimeras clearly show that ANA production is not determined by age related changes in radiosensitive, hemopoitic precursor cells, and that the CD4 T cells that promote ANA production is radioresistent. Thymectomy of young adult B6 mice leads to premature alterations in T cell homeostasis and ANA production, mimicking the conditions observed in old mice. Our findings indicate that a disturbed T cell homeostasis is closely associated with and likely to drive the onset of some autoimmune features.

Introduction

It is well established that the functional activity of the immune system declines with age [1]. The fact that mortality and morbidity during annual influenza epidemics increases in the elderly population is a clear example of this [2]. Moreover, influenza vaccinations are only marginally protective for the elderly [3, 4]. Yet, another example is the reactivation of the *Varicella zoster* virus. The frequency of this reactivation increases in individuals after the fiftieth year [5] and is thought to be due to a generalised decreased memory responses to chronic infections. In addition, some malignancies are more often found among the elderly [6]. For multiple myeloma the median age of diagnosis is 66 years and only 2% of all patients are younger than 40 years of age. The increased incidence of this plasma cell tumor and of certain other malignancies with age might suggest that in these cases immune surveillance is effective in young individuals, but declines with age.

The decline in immune competence is thought to be due to an age-related decrease in production of both B and T cells. We and others have shown that in both mouse and man, B cell production deceases with age [7-9]. Thus, the frequency of mouse bone marrow (BM) derived pro-B cells, that can be grown on stromal cells in the presence of IL-7 decreases with age: from around 1 in 50 at 2 weeks of age, whereas these drop to 1 in 500-1000 at 8 months of age. Similar findings were reported by others using FACS analysis of pro-B cells in BM [10]. In the BM of man, the decrease in B cell generation with age is even more striking, in that pro- and pre-B cells are practically undetectable by FACS in individuals older than 50 years of age [7, 8]. The finding, that the peripheral B cell repertoire in adults consist to a large extent of memory B cells (reviewed in [11]) might therefore be due in part to the fact that the turnover of

mature B cells, including memory B cells, is slow or even absent, since they do not have to compete for space with newly formed B cells from the BM.

The generation of T cells also decreases with age [12, 13]. Thus, the number of thymocytes and epithelial cells dramatically decreases with age. The mechanisms underlying this thymic involution are not understood. As a consequence of this the generation of new, naïve T cells is very low and therefore, replacement of mature peripheral T cells by newly formed ones is also very low. Most peripheral T cells found in an older individual have a memory phenotype [14, 15]. This shift in populations from a predominance of naïve cells to a predominance of memory cells with age is thought to be due to cumulative exposure to foreign antigens as well as the homeostatic proliferation of T cells in response to reduced thymus output.

While the generation of naïve T and B cells decrease with age, the incidences of autoimmunity and diseases increase in elderly persons. Autoantibody production to nuclear antigens (ANA's) [16, 17] and other autoimmune symptoms [18] are more frequently found in elderly individuals, and autoimmune diseases such as rheumatoid arthritis, Sjögren's syndrome and giant cell arteritis occur and/or peak in the second half of life [19-22]. The cellular mechanisms underlying the development of these auto-antibodies and diseaes are not understood.

Here, we studied inbred strains of mice for their capacity to develop autoimmune symptoms with age. We found that B6 mice develop autoimmune symptoms similar to those seen in elderly humans and began to study the underlying mechanisms.

Results

High incidence of potential autoimmity in aged C57BL/6 mice

We determined the IgG anti-nuclear antibody (ANA) titers in the sera of a cohort of young (2 – 3 months of age) and old (8 – 12 month of age) B6 mice. In the group of young B6 mice (n=14) IgG ANA were undetectable (Fig.1A). However, 85% (39 out of 46 mice) of old B6 mice were ANA positive and 20% (8 mice) of these had ANA titers of over 1 in a 1000 (Fig.1A). Histological analyses of spleen sections revealed the presence of large numbers of germinal centers in older mice (Fig.1B). Since high IgG autoantibody titers can be accompanied by the deposition of immune complexes in the kidney, cryosections of kidneys from old and young B6 mice were compared. As shown in Fig. 1C, IgG was readily detectable in the glomeruli of old mice but was undetectable in young mice (Fig.1D). The deposition of IgG in the glomeruli of old mice did not result in proteinuria (data not shown).

In man, the incidence of Sjögren's syndrome also increases with age [23]. Sjögren's syndrome is characterized by T and B cell infiltrations in the salivary glands. In order to test whether aging B6 mice also develop a Sjögren-like syndrome, immuno-histological analysis of salivary glands was performed. As shown in Fig. 1E, infiltrations of mononuclear cells were readily detectable in salivary glands of old B6 mice, but undetectable in the glands of young mice (data not shown). Immunofluorescence analysis revealed, that these infiltrates mainly consisted of T and B cells (Fig.1F).

Thus, aging B6 mice develop autoimmune features similar to aging humans. Therefore, analyses performed with these mice might unravel the mechanisms underlying the development of autoimmune features and eventually autoimmune diseases in elderly humans.

Kinetics of IgG ANA formation in various normal mouse strains

In the previous section, we have shown that around 80% of 1-year-old B6 mice had high IgG ANA titers in their blood. In order to determine the onset of IgG ANA formation, we determined the serum titers of these antibodies in a cohort of B6 mice (n=20) over time. As shown in Fig. 2A, the first ANA positive B6 mice were found at week 20 of age and their number steadily increased with time, reaching 90% of the mice after 1 year of age. IgG ANA titers in these B6 mice at 1 year of age were similar to those described in Fig. 1A. Different results were obtained when the same analysis was performed in other mouse strains. In 20 BALB/c mice IgG ANA were found at lower titers (1/160) in 1 mouse at week 36 and in 4 mice at 1 year of age, In the DBA/2 strain none of 15 mice developed IgG ANA during more than one year of observation. Taken together, these findings indicate that B6 mice are genetically prone to spontaneously develop IgG ANA, whereas BALB/c and especially DBA/2 mice are much more resistant to the development of this autoimmune feature.

The high incidence of IgG ANA in B6 mice and the absence of these in DBA/2 mice prompted us to follow a large cohort (n=60) of BDF_1 mice for the presence of IgG ANA in their sera. As shown in Fig. 2A, the first ANA positive mice were found at week 32 of age when already 40% of the parental B6 mice were ANA positive. By week 60, 25% (15 out of 60) of the BDF1 mice were IgG ANA positive. The ANA titers of these mice varied from 1 in 40 to 1 in 160, i.e. were relatively low. Thus, the incidence and severity of IgG ANA formation in aging mice is strongly dependent on genes that are present in B6 mice, but are absent in DBA/2 mice.

The IgG ANA production in B6 mice is CD4 T cell dependent

The fact that the ANA in ageing B6 mice were of the IgG class indicates that ANA formation in these mice is a T cell-dependent process. We confirmed the crucial role of CD4 T cells by comparing IgG ANA production in normal B6 mice with that of B6 mice that were MHC class II $^{-/-}$ and thus deficient in $CD4^{+}$ T cells. As found before, practically all (18 out of 20) normal B6 mice had IgG ANA in their serum at 1 year of age (Fig. 2B). In contrast, only 1 out of 14 MHC class II $^{-/-}$ B6 mice developed IgG ANA at a very low serum titer of 1/20.

The IgG ANA is not dependent on age related changes in radiosensitive hematopoietic progenitor cells

In a first approach to determine in which cells age related changes are required for IgG ANA production we generated a set of 4 syngeneic bone marrow chimeras. Thus, old ANA positive and young ANA negative B6 mice were lethally irradiated and reconstituted with T cell depleted bone marrow (BM) cells derived from either old ANA positive or young ANA negative B6 mice. At 6-8 weeks after BM reconstitution, IgG ANA titers were determined in the various chimeras. All chimeras in which the host was old produced IgG ANA irrespective of the BM origin. The ANA titers in these mice varied from 1 in 40 to 1 in 640 (Fig. 3A). In contrast, no IgG ANA was detectable in the chimeras in which the host was young. The lack of IgG ANA formation in young hosts reconstituted with BM cells from young or old donors indicates that age related changes in hematopoietic progenitor cells are insufficient for ANA formation. The fact that IgG ANA production depended on the age of the host, but not the age of donor cells indicates that the relevant age related change must occur in radioresistant cells of the host.

CD4 T cells involved in IgG ANA formation in aging B6 mice are radioresistant

The radioresistant cells in old B6 hosts that are crucial for IgG ANA production could be nonhematopoietic cells, for example cells which express the relevant nuclear antigens in an immunogenic form only with age. However, the role of radoresistant hematopoietic cells could not be excluded. We and others have shown that hematopoietic cells, especially T cells, can be radio-resistant [24-26]. Therefore, ANA production in old B6 hosts could be driven by radio-resistant T cells of these mice. In order to analyze this possibility we generated another set of chimeras. ANA positive B6 mice were lethally irradiated and reconstituted with either B6 MHC class $II^{-/-}$, B6 *RAG-2*$^{-/-}$, B6 μMT or B6 *CD3*$^{-/-}$ BM cells. At 6-8 weeks after reconstitution IgG ANA titers were determined. No IgG ANA was detectable in B6 *RAG-2*$^{-/-}$ and B6 μMT chimeras, indicating a complete ablation of the host B cell compartment due to irradiation (Fig. 3B). Only one out of five chimeras receiving MHC class $II^{-/-}$ BM showed a low titer of IgG ANA. However, all 6 chimeras reconstituted with B6 *CD3*$^{-/-}$ BM cells showed very significant IgG ANA titers (Fig.3B). Since the B6 chimera-hosts were $CD45.2^{+}$ and the B6 *CD3*$^{-/-}$ BM cells were $CD45.1^{+}$ we were able to identify and analyze host derived hematopoietic cells in these chimeras. As shown in Fig. 4A about 14% of the cells found in the spleen of these chimeras were of host origin and practically all of those (96%) were T cells. In contrast, all B cells were derived from the transferred B6 *CD3*$^{-/-}$ BM cells (Fig. 4B). In order to directly show that the host derived T cells were involved in the IgG ANA formation, the $CD4^{+}$ cells of these chimeras were sorted and 2 x 10^5 transferred into non-irradiated young $CD45.1^{+}$ B6 *CD3*$^{-/-}$ or $CD45.1^{+}$ wild type B6 mice. At 5 weeks after transfer, all 5 of the $CD45.1^{+}$ B6 *CD3*$^{-/-}$ mice were IgG ANA positive (titers varying from 1 in 40 to 1

in 320) and 2 out of 5 CD45.1^{+} B6 mice were IgG ANA positive (titers 1 in 40 and 1 in 160) (Fig. 4E). Thus, the radio-resistant CD4 T cells found in old B6 mice could, upon transfer, induce IgG ANA formation in young B6 hosts. As shown in Fig. 4C, transferred cells were readily detectable in the injected T cell deficient, young CD45.1^{+} B6 *CD3*$^{-/-}$ mice, i.e. around 7% of the spleen cells were CD45.2^{+} CD4 T cells. In the young CD45.1^{+} B6 recipient which are not T cell deficient, the transferred CD45.2^{+} CD4 T cells were also detectable, albeit at very low numbers only, i.e. 0.1% of the spleen cells were CD45.2^{+} CD4 T cells (Fig. 4D). The low numbers of transferred cells in the normal B6 recipients could be due to competition by recipient T cells or their elimination by a response of the recipient T cells to the CD45.2 antigen. In any case, the low survival of transferred cells is the most likely explanation for the absence of IgG ANA in sera of 3 out of 5 recipients (Fig. 4E). However, a specific suppression of IgG ANA by recent thymus emigrants cannot be excluded.

Disturbed T cell homeostasis seems to underlie the formation of IgG ANA in old B6 mice.

It is well established that the thymus involutes with age. In our animal unit, B6 mice at 2 months of age have around 140 x 10^6 thymocytes, whereas this number declines to 40 x 10^6 at one year and to 20 x 10^6 at two years of age. Therefore, the peripheral homeostasis of T cells in old mice is different from that in young mice and thus might be responsible for the onset of IgG ANA formation in old B6 mice. Another explanation for the observed autoimmune phenomena might be that negative selection of T cells in an old thymus is impaired. To discriminate between these two possibilities, we followed the IgG ANA formation in a cohort of B6 mice (n=20), that

had been thymectomized (Tx) at 5 weeks of age, i.e. well before any IgG ANA production had commenced and compared them to non-thymectomized B6 mice. In the case of disturbed T cell homeostasis, Tx B6 mice should generate IgG ANA much earlier in life than normal B6 mice. However, if impaired negative selection of T cells in the old thymus is the cause of IgG ANA formation, Tx B6 mice should not generate IgG ANA at any age. We found that B6 mice thymectomized at the age of 5 weeks start to develop IgG ANA already at week 12 of their life (Fig. 5A). At week 15, 90% of the thymectomized mice, but only 5% of non-thymectomized B6 mice, have significant levels of IgG ANA in their circulation. In a first attempt to understand the IgG ANA production in old normal and young thymectomized mice we analyse the peripheral T cell compartment in these mice.

6-8 week old B6 mice have 10-15 x 10^6 $CD4^+$ T cells in their spleens of which more than 80% have a naïve ($CD62L^+$) phenotype (Fig. 5B). 8 months old normal B6 mice have around the same number of splenic $CD4^+$ T cells, but more than 60% of those have a memory ($CD44^+$) phenotype. 18-20 weeks after thymectomy, spleens contain 8-10 x 10^6 $CD4^+$ T cells of which 90% display a memory phenotype. Both old normal and thymectomized young B6 mice show a dramatically increased proportion of $CD4^+$ T cells expressing the transcription factor Foxp3, and thus are regulatory T cells (Tregs) (Fig. 5C). Likewise, old as well as young thymectomized mice showed increased proportions of $CD4^+$ T cells expressing the cytokines IFN-γ and IL-2 (Fig. 5D and E).

Thus, the impaired homeostasis induced by thymectomy generates a pool of peripheral T cells phenotypically resembling that found in old mice.

Discussion

We report herein that in ageing mice, like in elderly humans, development of features characteristic of autoimmunity is a common occurrence [1]. Thus, we find that virtually all 1 year-old mice of the C57BL/6 (B6) strain spontaneously show high titers of IgG anti-nuclear antibodies (ANA). A majority of them also display infiltrates of T and B lymphocytes in their salivary glands (Sjögren-like syndrome) and some 10% have deposits of IgG in kidney glomeruli. Despite all these signs of autoimmunity, like in most elderly humans, these ageing animals remain healthy. Like in many animal models of spontaneously developing autoimmune disease, the time of onset and the severity of symptoms is highly variable, even among genetically-identical individuals. Some of our B6 mice start to produce IgG ANA already at 13 weeks of age, whereas others, even of the same litter, kept in the same cage, only start ANA production at week 60 or later. Viral transmission would seem a very unlikely cause of this since mice with or without IgG ANA were found in the same cage. The reasons for this variability among genetically identical individuals are not known.

The incidence of IgG ANA production is reduced (30%) and delayed (week 30) in our ageing BALB/c mice and completely absent in the MHC haplotype identical DBA/2 mice, which are known to generate normal immune responses to immunization [27]. Thus, the B6 strain is highly susceptible for the spontaneous development of IgG ANA autoantibodies. In some cases where IgG ANA could be detected in serum, deposits of IgG were observed in the glomeruli of kidneys. However, we never observed severe kidney damage with leaking of proteins into the urine. We do not know whether these deposits represent IgG antibodies directly binding to the

basement membranes, or are immune complexes, perhaps of the very ANA's in complex with their nuclear antigens.

Interestingly, previous studies have shown that B6 mice congenic for lupus susceptible loci rather often develop spontaneous pathological autoimmunity [28-30]. Thus, little genetic change is needed to transform B6 mice producing harmless IgG ANA into an autoimmune disease prone mouse strain.

As expected, the IgG ANA production is T cell dependent, since B6 mice deficient in MHC class II expression almost never show any IgG ANA production. Moreover, the findings that lethally irradiated old B6 mice reconstituted with wild type or CD3 deficient BM develop IgG ANA, whereas those reconstituted with BM from MHC Class II deficient mice generally do not, strongly indicates that cognate interaction between T and B cells is required for IgG ANA production.

When the highly permissive B6 strain is crossed to the non-permissive DBA/2 strain, the resulting F1 animals show a much-delayed onset of IgG ANA production with only a 25% penetrance at 60 weeks of age. Thus, it seems, that the DBA/2 genotype exercises some kind of dominant suppression over the permissive B6 genotype in its ability to produce IgG ANA. The nature of this suppression remains to be determined.

Since the T cell dependent IgG response to ANA is so dramatically different between young and old mice, and since it is observed in young thymectomized mice, we characterized the peripheral T cell compartment in young and old B6 mice as well as in thymectomized mice. We find that memory-type $CD4^+$ T cells accumulate in the spleens of both older and thymectomized animals , and that these T cells produce the pro-inflammatory cytokines IFN-γ and IL-2. As these cells survive lethal irradiation in old B6 mice, they can be isolated from such mice and shown to induce IgG ANA production upon transfer to young mice. The T cell compartment of thymectomized

young mice also consisted of increased numbers of memory-phenotype $CD4^+$ T cells, similar to that of old mice. In old, as well as in thymectomized mice, an increase in Treg's was observed. The elevated expression of IL-2 might drive antigen independent proliferation of Treg's. Thus, the IgG ANA production in old B6 mice and young thymectomized B6 mice is associated with the disturbance of T cell homeostasis that is caused by the reduction or lack of thymic output of naïve T cells. However, it should be noted that not in all situations in which T cell homeostasis is disturbed, this results in the development of autoimmune features. Thus, preTα deficient mice display a severely disturbed T cell development in the thymus and a largely reduced peripheral T cell compartment [31, 32]. However, these mice do not show early signs of autoimmunity (own unpublished observation). Yet, another example is the Coronin 1 deficient mouse, which has a disturbed and reduced peripheral T cell compartment but does not show autoimmunity [30, 33]. To the contrary, a Coronin 1 deficiency in MRL.lpr mice blocks, to a large extent, the development of Lupus-like disease in these mice [30].

Two key questions remain to be answered: what is the nature of the T cell dependent antigens and how is tolerance to these antigens broken? It is obvious from our experiments that the antigens relevant for ANA production is present already in young B6 mice in an immunogenic form, because ANA responses can be provoked in young mice by thymectomy (Fig. 5) or by transfer of radioresistant CD4 T cells from old mice into young recipients (Fig. 4) and by the induction of a chronic graft-versus-host disease in young mice (data not shown). The requirement of a cognate interaction between T and B cells for obtaining an ANA response argues that the antigen has to be expressed by B cells. In SLE patients the nuclear antigens recognized by ANA include single and double stranded DNA. We also observe some 40% of our IgG

ANA from ageing B6 mice to be specific for DNA (data not shown). The fine specificity of ANA in the serum of SLE patients and in the disease causing deposits of immune complexes in the kidney have only partly been determined [34, 35]. The antigenic determinants that are recognized by the IgG ANA inducing T cells are not at all known. However, as pointed out above, an age dependent appearance of these antigens cannot explain the age dependency of IgG ANA formation.

How is tolerance of CD4 T cells involved in IgG ANA production broken in old mice? Various other mouse models for autoimmune disease, and in particular ANA production in strains of mice developing a Lupus-like disease, have identified different genetic loci responsible for the breaking of T cell tolerance [30, 36-39]. How these loci operate in ageing, normal B6 mice remains to be determined.

In general, tolerance of T cells to self antigens can be broken by the failure of negative selection in the thymus, or by the failure of suppression in the periphery. We exclude the age dependent failure of negative selection in aged mice as a cause of ANA production by showing that ANA production does occur in young thymectomized mice. On the other hand a failure of suppression is consistent with our observation that IgG ANA production is reduced in a cross between ANA producing B6 mice and DBA/2 mice which do not produce ANA. However, the mechanisms underlying the suppression of IgG ANA responses remains to be determined. Loss of suppression of ANA responses by a loss of Treg in old mice is unlikely, since the number of these cells increases as a result of reduced thymic output. However supression of ANA responses by Treg or other cells that are recent thymic emigrants in young mice and loss of this suppression in old mice cannot be excluded as the cause of IgG ANA production in old mice.

The major cause of a disturbed T cell homeostasis in ageing animals is a decreased thymic output of naïve T cells due to the process of thymic involution [40]. It is possible, that the variability of onset of ANA production which we observe between animals of the same strain and particularly between different strains has its origin in differences among animals regarding the onset of thymic involution. Our findings suggest that the incidence and/or severity of autoimmune disease may be reduced by treatment modalities that improve thymic output and prevent or delay thymic involution. Such therapies may be initiated in genetically susceptible individuals or in individuals with very early signs of autoimmunity.

Materials and Methods

Mice

C57BL/6 (B6), BALB/c, DBA/2, (C57BL/6 x DBA/2)F1 (BDF1), MHC class $II^{-/-}$ (C57BL/6 $IA\alpha^{-/-}$) [41], B6 *RAG-2*$^{-/-}$ [42], B6 μMT (C57BL/6-Igh-6^{tm1Cgn}) [43] and B6 *CD3*$^{-/-}$ [44] mice were bred under SPF conditions in our animal unit. The State veterinary authorities of Basel (Kantonales Veterinäramt, Basel-Stadt) have approved all animal experiments.

Antibodies and flow cytometric analysis

FITC-, PE-, APC- or biotin-conjugated mAb specific for CD4 (L3T4), CD8α (53-6.7), CD69 (H1.2F3), CD44 (IM7), CD62L (MEL-14), anti-TCR β-chain (H57-597), were purchased from BD Bioscience (Allschwil, Switzerland). Streptavidin, conjugated to PE, APC or PE/Cy7 were also purchased from BD Bioscience. Anti-FoxP3 (FJK-16s), anti-IFN-γ (XMG1.2) and anti-GM-CSF (MPI-22E9) were purchased from eBioscience. The anti-IL2 (S4B6), anti-CD45.1 (A20-1.7), anti-CD45.2 (104-2.1), anti-IgM (M41) and anti-CD90 (T24) mAb's were purified from hybridoma supernatants and labeled with biotin, FITC or A647 in our laboratory by standard methods. Flow cytometry was performed using a ^{TM}FACS Calibur (BD Biosience) and sorting using the ^{TM}FACS-ARIA (BD Biosience). Data were analyzed using the ^{TM}Cell Quest Pro Software (BD Bioscience).

Immunohistochemical and histological analyses

Spleens, kidneys and salivary glands were snap frozen on dry ice and embedded in OCT-compound (Sakura, Zoetermeer, NL), and 5 μm sections were prepared. Sections were fixed in acetone for 10 min and then air dried for 60 min. To analyze B and T cell organization in spleen and to determine the presence of germinal centers in spleen, sections were incubated with biotinylated anti-IgM (clone M41) and FITC

labeled anti-CD90 (T24) or with anti-IgM FITC (M41) and biotinylated peanut agglutinin (PNA) (Vector, Burlingame, CA) for 30 min. After 20 min of washing in PBS, anti-IgM and PNA binding were revealed with PE conjugated streptavidin (SouthernBiotech, Birmingham, AL). Fixed salivary gland sections were additionally stained with hematoxylin and eosin (H&E) according to standard procedures. Kidney sections were incubated with FITC labeled goat anti-mouse IgG mAb (Jackson Immunoresearch, Milan Analytica, La Roche, CH) to reveal IgG deposits in glomeruli.

IgG anti-nuclear autoantibody determination

IgG autoantibodies against nuclear antigens were detected by an indirect immunofluorescence technique (described in [25]), using a FITC labeled goat anti-mouse IgG (Jackson Immunoresearch, Milan Analytica, La Roche, CH); 5 μm cryosections of kidneys from RAG-2 deficient mice were used as substrate. The titer was defined as the highest serum dilution still giving a positive nuclear staining. Titers lower than 1/20 are denoted "not detectable".

PMA/ionomycine stimulation

Total lymphocytes were stimulated at 37°C during 4 hours using 1 μg/ml ionomycine (Sigma-Aldrich) and 5ng/ml phorbol-12-myristate-13-acetate (PMA) in the presence of 10μg/ml brefeldin A (Calbiochem). Cells were harvested and stained by standard intracellular staining procedure. In brief, surface staining was followed by a fixation step using 2% paraformaldehyde in PBS followed by intracellular staining in FACS buffer containing 0.5% saponin.

BM chimaera

Bone marrow (BM) was isolated from femur and tibia of donor mice. BM cell suspension was incubated with anti-CD4 (RL172) and anti-CD8 mAb (31M) prior to

complement depletion of T cells by incubating the sample with rabbit serum (Low-Tox®-M, Cederlane Laboratories Ltd. 1:20 in DMEM) for 30 min at 37°C. The procedure was followed by erythrocyte lysis buffer treatment. Six weeks or >8 months old B6 recipients were lethally γ-irradiated (900 rad) 4 hours prior to intravenous injection of 5 x 10^6 T cell depleted BM cells/mouse. BM chimaeras were analyzed in week 6 after BM reconstitution.

T cell transfer

Radioresistant host CD45.2$^+$ CD4$^+$ T cells isolated from spleens of old B6 *CD3*$^{-/-}$ BM chimaera (IgG ANA positive) were sorted and 2 x 10^5 cells were transferred into non-irradiated 6 week old CD45.1$^+$ B6 *CD3*$^{-/-}$ or CD45.1$^+$ wild type B6 mice. IgG ANA titers were analyzed 5 weeks after T cell transfer.

Thymectomy of B6 mice

At 5 weeks of age, mice were anesthetized and the thymus removed by suction through a small upper sternal incision. Completeness of thymectomy was verified in each animal by anatomical inspection at the time of sacrifice.

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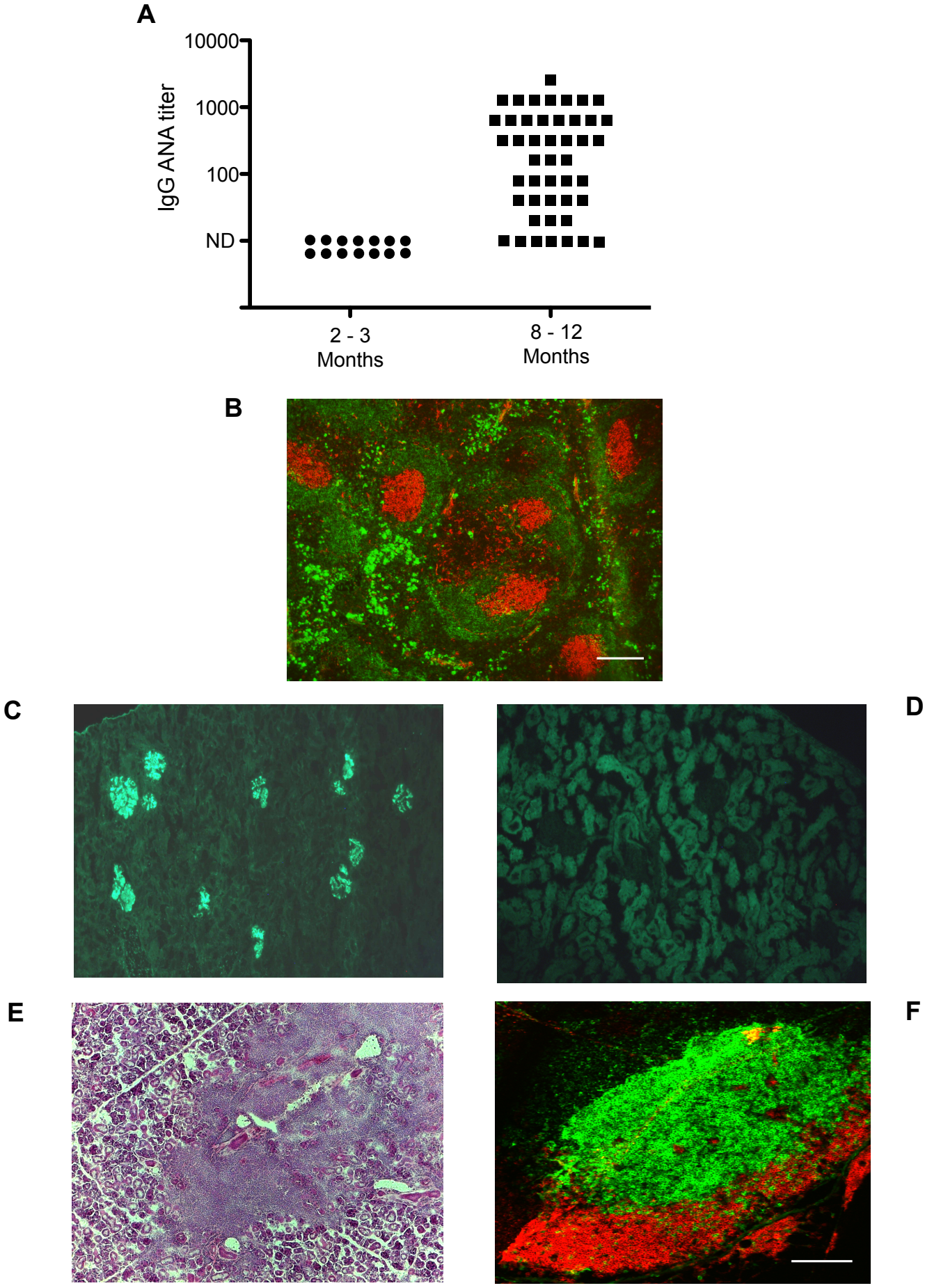

Figure 1. Old mice show high incidence of circulating IgG ANA, germinal center formation, and kidney depositions of IgG as well as development of lymphocyte infiltrates in salivary glands. Titers of IgG ANA in sera of young (to the left) and old (to the right) B6 mice (ND denotes not detectable) (A). Section of spleen from an old B6 mouse stained with PNA (red) and anti-IgM (green) antibody (B). Section of kidney from old (C) and young (B) B6 mice stained with FITC anti-IgG antibody (green). Section of salivary gland from an old B6 mouse stained with H&E (E) or anti-CD90 (green) and anti-IgM (red) antibodies (F) (scale bar: 150 μm).

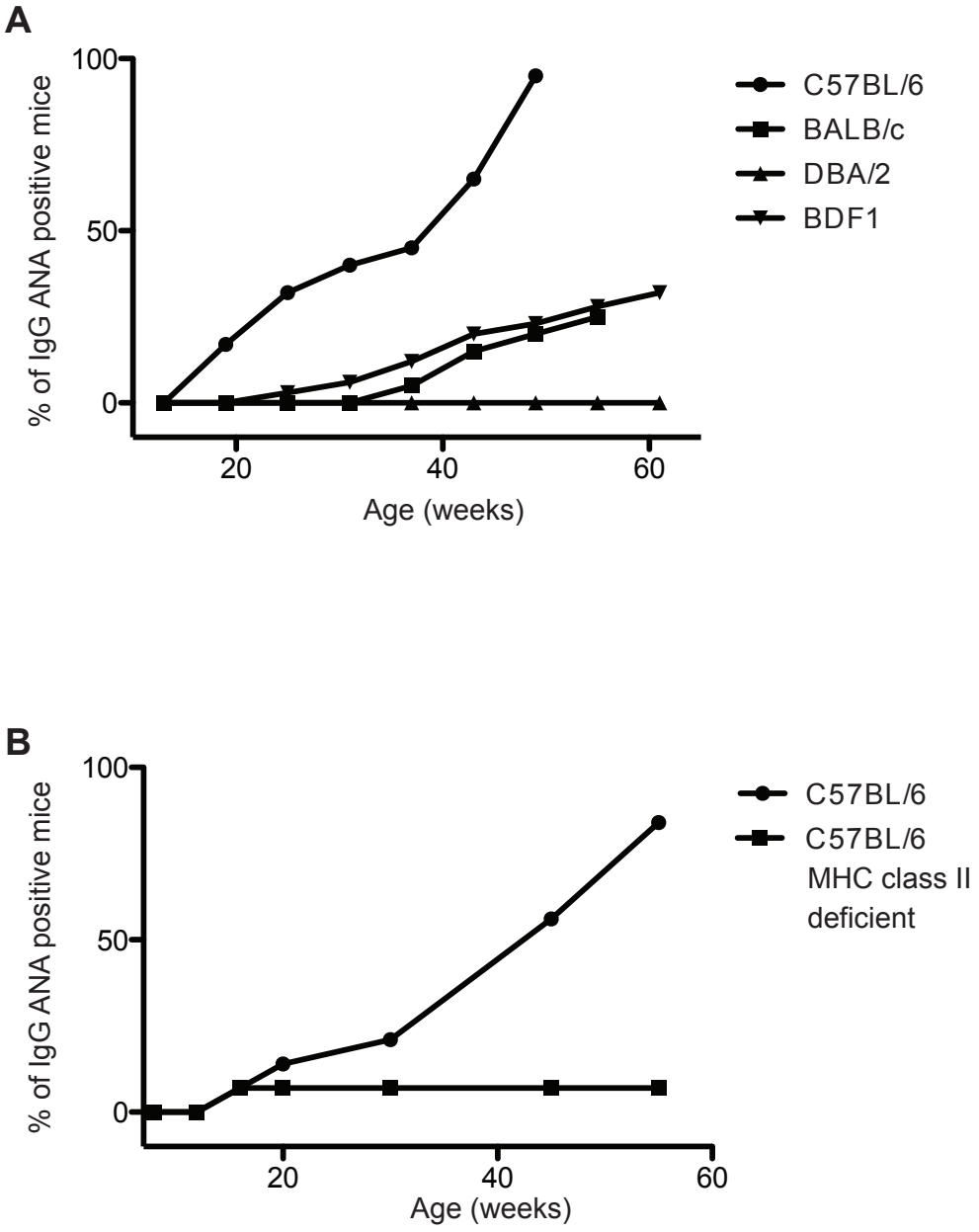

Figure 2. Kinetics of IgG ANA appearance in sera of different inbred mouse strains. Titers of IgG ANA were determined at indicated times after birth in various inbred mouse strains (A) as well as in MHC class II deficient mice (B).

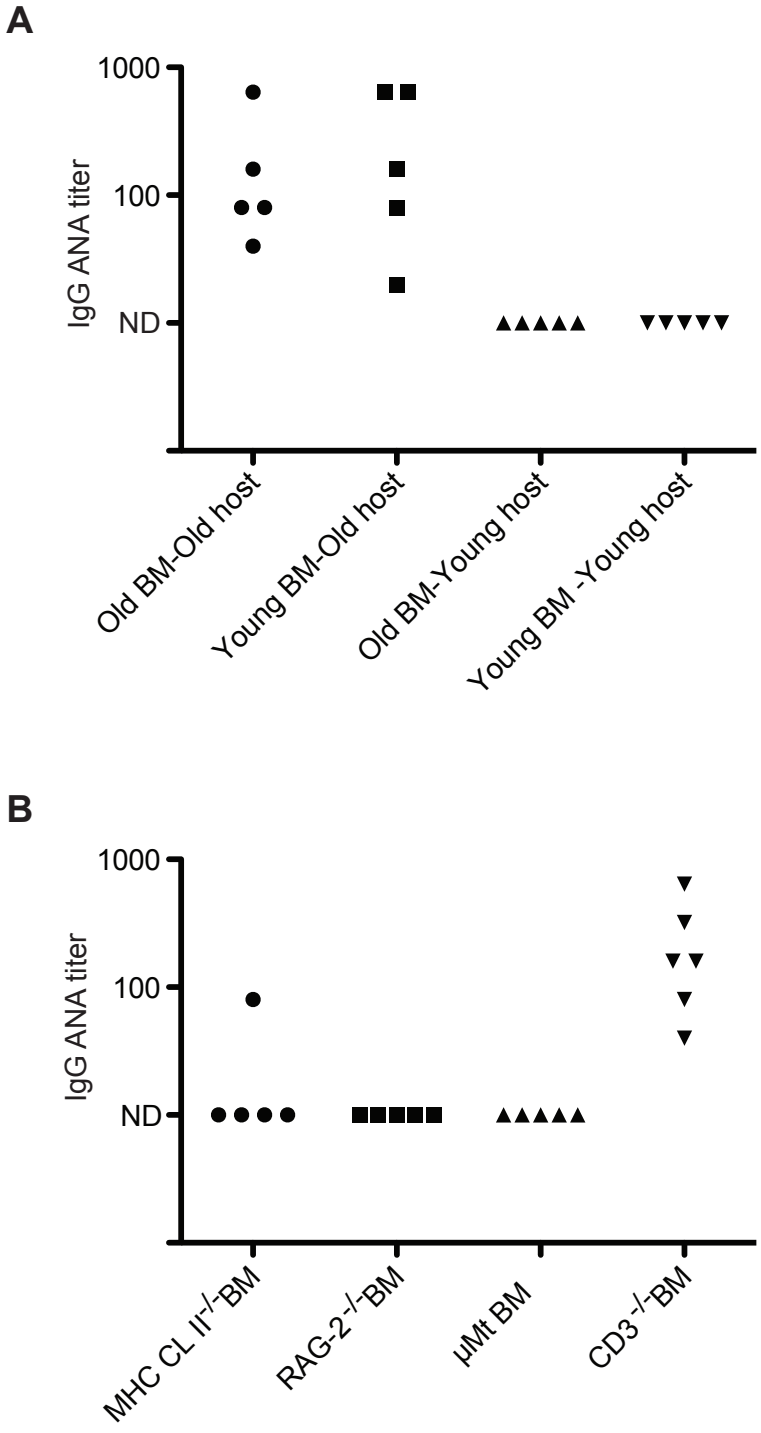

Figure 3. IgG ANA titers in BM chimeras. Young or old B6 host mice were lethally irradiated and reconstituted with T cell depleted BM from young or old B6 mice. Mice were bled 6-8 weeks after reconstitution and the ANA titers determined (A). Old ANA positive B6 host mice were lethally irradiated and reconstituted with T cell depleted BM from indicated donors. ANA titers were determined 6-8 weeks after reconstitution (B). ND denotes not detectable.

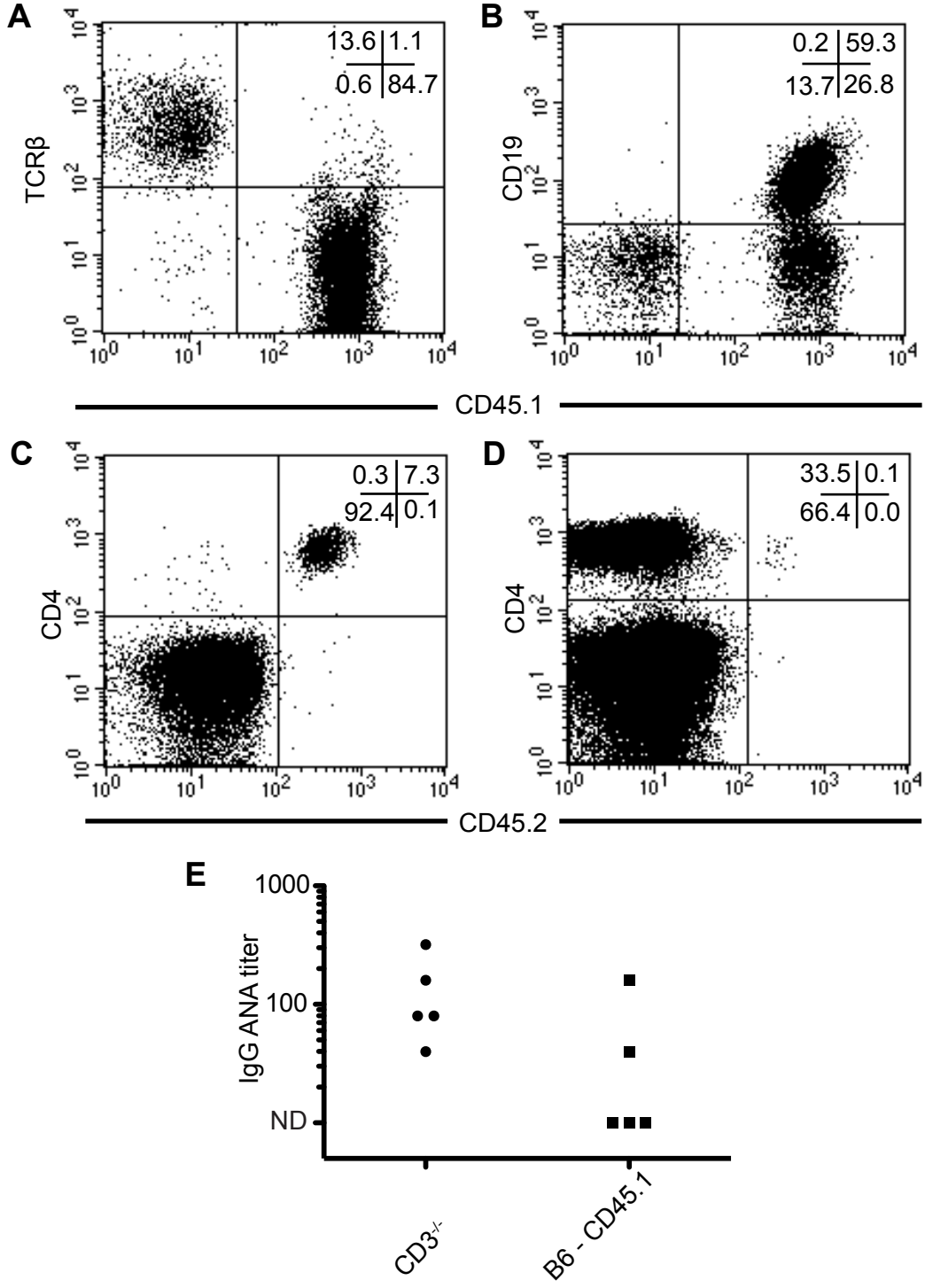

Figure 4. Radioresistant CD4+ T cells can transfer the capacity for ANA production in young hosts. Old ANA positive B6 host mice were lethally irradiated and reconstituted with BM from CD3-/- donor mice. 8 weeks after reconstitution spleens were analyzed by FACS for host derived, CD45.1 negative T cells (TCRβ+) (A) or donor derived CD45.1+ B cells (CD19+) (B). Host derived (CD45.2+) T cells were sorted and transferred to non-irradiated CD3-/- (C) or wt (D) mice. 5 weeks after transfer, recipient mice were bled and their serum ANA titers determined (E) and their spleens analyzed for donor derived (CD45.2+) CD4 positive T cells (C and D). ND denotes not detectable.

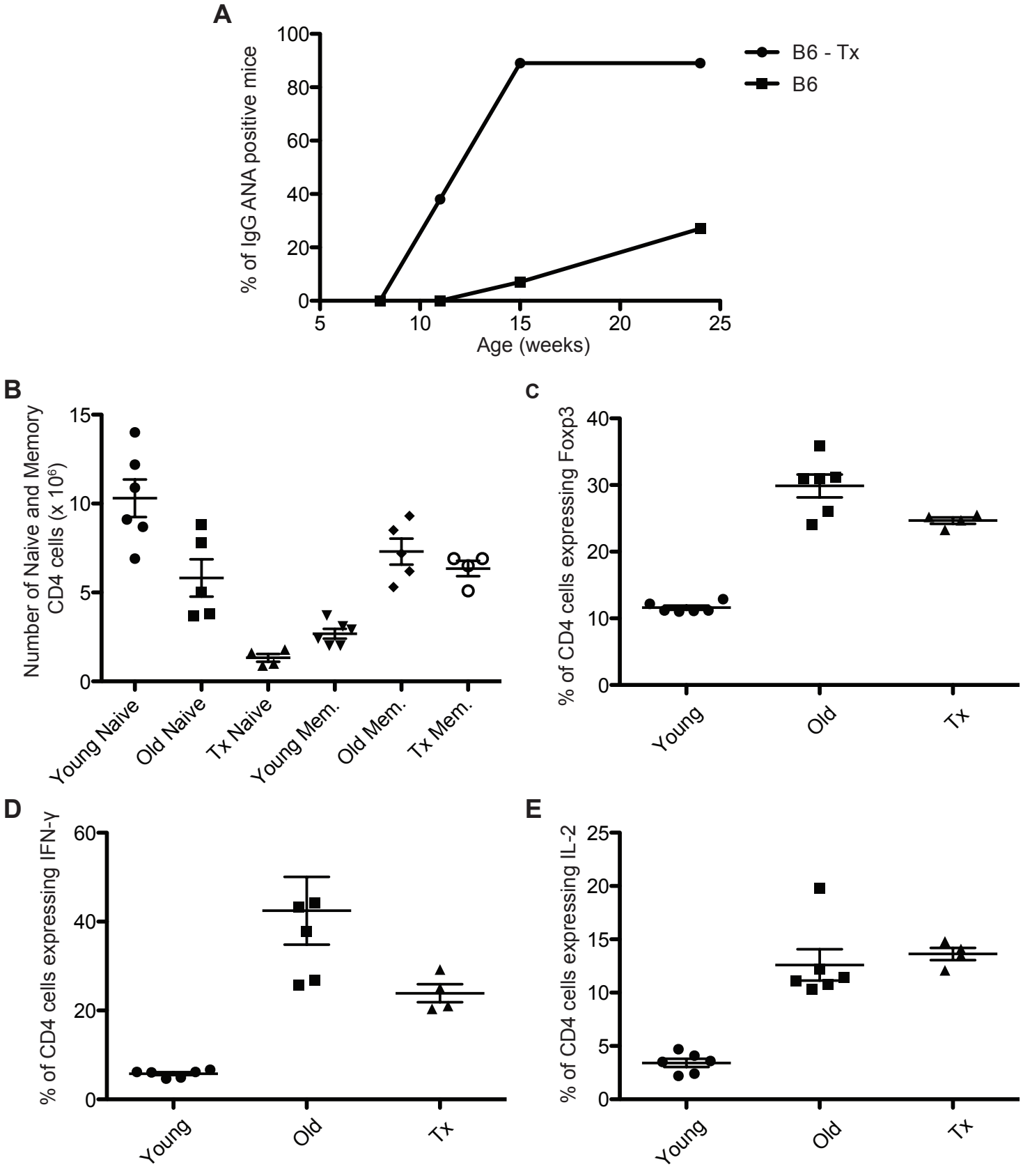

Figure 5. Adult thymectomy enhances IgG ANA production. 5-6 week old B6 mice were thymectomized (Tx). At indicated times, sera were collected and analyzed for IgG ANA (A). 18 weeks after thymectomy, spleens were analysed for $CD4^+$ T cells with memory ($CD44^+$) or naïve ($CD62L^+$) phenotype. In the same FACS analyses, spleens from young (8 weeks) and old (> 8 months) normal B6 mice were similarly analyzed (B). The percentage of $CD4^+$ splenic T cells expressing Foxp3 (C), IFN-γ (D) as well as IL-2 (E) was determined by FACS as described in materials and methods.

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Curriculum vitae

Personal data

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PhD study

Oct. 09 –Oct. 13 (exp.)	PhD study in Developmental and Molecular Immunology, DBM University of Basel in the research group of Prof. Antonius Rolink PhD thesis: "Development of autoimmune-like disease due to a lack of T cell tolerance" (running titel)

Studies (1 excellent to 6 unsatisfactory)

Sept. 05 – June 09	main study period in Technical Biology at the University of Stuttgart: - diploma thesis "Immunoliposomes targeting EGFR and HER2 receptor – an approach for HER2 receptor silencing in an adherent cell model" - final examination: immunology and antibody engineering (main subject, 1.0), bioprocess engineering (subsidiary subject, 1.0), zoology (marine biology, subsidiary subject, 1.0), biochemistry (1.3)
Sept. 03 – July 05	basic studies in Technical Biology at the University of Stuttgart, final average: good

International experience

Oct. 09 – DBM, expected Oct. 13	PhD study in Developmental and Molecular Immunology, University of Basel (Switzerland)
July 07 – Dec. 07	working on a project at Integrin (Scotland) to pass my student research project "Development of a real-time PCR detection method for *Bacteroides* spp. in shellfish and water samples" (1.3)

Skills / competences

Methods:

- flow cytometry of lymphoid cells
- culture of freshly isolated lymphoid cells, tumor cell lines
- molecular biology techniques (PCR, qPCR, rtPCR, RNA/DNA extraction, southern blot, cloning)
- functional T cell assays *in vitro*, MLR, FTOC
- ELISA of mouse serum, tissue culture supernatants
- protein expression in bacteria and affinity chromatography purification
- immuno-liposome generation
- confocal microscopy of fluorescent labelled tissue sections
- experimental animal work (sacrificing mice and collecting organs):
  - LTK Module 1 (Introductory Course in Laboratory Animal Science, accredited by FELASA, University of Zürich 2010)
  - course in experimental animal work (accredited by the Society of Laboratory Animals (GV-SOLAS) (10h lecture, 30h practical exercise, University of Stuttgart 2008)

Activities:

- organization of the "Immuno-PhD students club" for PhD students working in the field of immunology at the DBM/ZLF (University of Basel)
- preparation of experiments and assistance of undergraduate students in the annual "Blockkurs" (laboratory course in immunology of the University of Basel)
- training of undergraduate students in different laboratory technics during their master thesis

Computer skills:

- MS Office, ImageJ, GraphPad Prism, Adobe Illustrator, FlowJo, EndNote

Foreign language:

German, mother tongue
English, fluent in speaking and writing
French, good knowledge in speaking and writing

Publications / manuscripts in preparation

- **Nusser, A.**, Swee, L.K., Curti, M., Kreuzaler, M., Rolink, H., Terracciano, L., Melchers, F., Andersson, J. & Rolink A.The amount of self-antigen determines the effector function of murine T cells escaping negative selection. (under revision)
- **Nusser, A.**, Nuber, N., Wirz, O., Rolink, J., Andersson, J. & Rolink, A. The development of autoimmune features in aging mice is closely associated with alterations of the peripheral CD4 T cell compartment. (running titel)
- **Nusser, A.**, Gehre, N., von Muenchow, L., Tussiwand, R., Engdahl, C., Capoferri, G., Bosco, N., Ceredig, R. & Rolink, A. Establishment of a stromal cell free culture system that allows the long-term propagation and proliferation of pro T cells, which can be used for the *in vivo* reconstitution of the T cell compartments (running titel)
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Poster presentation

- EuroThyme Rolduc, Amsterdam (NL) 2012
- Immune Tolerance and Autoimmune Disease, Cambridge (UK) 2012
- 24th Meeting of the Swiss Immunology PhD students, Schloss Wolfsberg (CH) 2012 (oral presentation)

References

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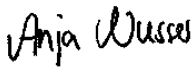

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